A SPECTROPHOTOMETRIC ANALYSIS OF BLOOD AND MUSCLE HEMOGLOBIN SOLUTIONS

by

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INTRODUCTION

In certain commercial channels, dark-red lean beef is believed to be tougher and less palatable than that of a bright cherry-red color. Therefore, a prejudice has arisen against dark cutting beef which has resulted in discrimination by consumers and retailers, resulting often in a financial loss to the packer and consequently a lower price paid for certain cattle. Many explanations of dark cutting beef have been given, some of which are breed, color of the cattle, age, sex, feed used and others. Enough data is available to practically eliminate most of these except the feed used, which explanation has received more attention than any other. Grass as a feed is blamed more than any other single feed.

In an attempt to determine whether or not grass fed animals cut dark, a number of the experiment stations have done considerable work on animals given different feeds. Samples of various muscles were taken and a chemical and physical analysis made. Included in the physical analysis was a color comparison test. The color test was made by the use of a Munsell color wheel in which varying amounts of red, yellow, white and black could be combined until the color of the spinning disk compared favorably with that of the meat under observation. The exposed area of each color on the

disk is then measured and recorded. The greater the number of units of black in a reading, the darker the color of the sample, or the greater the number of red units present in a reading, the brighter the red of the sample. This method merely gives a comparison of the color of one sample relative to that of another and gives no information as to the identity or the relative amounts of the pigments present. However, by this study it was found that the color of meat brightens very materially after cutting, quite rapidly at first and continuing to brighten for about three hours.

It is well known that there are two red pigments in muscle but so far no method has been given for the determination of the relative amounts of the two in a mixture. These two pigments, muscle and blood hemoglobin, have similar absorption bands in the visible but they differ slightly in position. The purpose of this thesis was to make a spectrophotometric study of the two red pigments found in blood and muscle. In making this study it was proposed to determine the points of maximum and minimum absorption of light, the ratio of the densities at these points and from this information devise if possible, a method for the determination of the relative amounts of these pigments in a sample of meat. This information might aid in solving the problem of what causes dark cutting beef.

It is the absorbed light which is of interest to chemists, more particularly the absorption of certain bands of color. The observation of the location of particular absorption bands and the amount of the incident light absorbed, enables him to identify substances,to discriminate between them, to estimate concentrations of solutions, and to observe the results and approximate rate of chemical changes. If the eye is replaced by a quartz spectrograph, an additional spectral range can be observed which is considerably greater than that visible to the eye. The quartz spectrograph is very useful in disciminating between different substances whose spectra in the visible do not differ to any marked degree and also can be used for determining purity of solutions.

When light passes through any homogeneous transparent medium, it emerges diminished in amount. Part of the light may be scattered at the surfaces, part scattered in the interior and part regularly reflected at the surfaces. The rest of the light which is lost is said to be absorbed. It may be transformed into heat or into fluorescent or phosphorescent light of different wave-lengths from its own, or it may cause ionization or chemical action.

Since only the absorbed part of the light is of significance in this kind of work, allowance must be made for

the other lost light in taking measurements. This is compensated for by arranging that another non-absorbing substance with reflecting surfaces similar to that to be measured is used for comparison.

The two principal laws of absorption are those of Lambert and Beer. Lambert's law states that the proportion of light absorbed by a substance is independent of the intensity of the incident light. This law according to Twyman (1) is rigidly true. In defining extinction coefficient, Bunsen and Roscoe assumed only Lambert's law: "Let I_0 be the intensity of the incident light which enters the medium, I the intensity remaining after its passage through a path length d, and $1/\epsilon$ the path length, passage through which reduces the light intensity to 1/10th; then

$$I = I_0 \cdot 10^{-d\epsilon}$$

where ϵ, a constant depending on the medium, is called by us the 'extinction coefficient' d is to be taken in centimeters." The extinction coefficient is the reciprocal of the thickness which is necessary to reduce the light intensity to 1/10th of its incident value. Or considering I_0 as unity, the equation may be written,

$$\epsilon = -\frac{\log I}{d}$$

Beer's law states that if an absorbing salt is dissolved in a non-absorbing liquid, its absorption of a beam of homogeneous light depends on the number of molecules of the absorbing substance which the beam of light passes through, that is, on the concentration of the solution.

Then

$$\frac{C_1}{C_2} = \frac{\epsilon_1}{\epsilon_2} = \frac{-\log I_1}{-\log I_2} = \frac{\log I_1}{\log I_2}$$

or the absorption of light by different concentrations of the same substance in the same solvent is directly proportional to the concentration if the depth is the same.

There is another term which is often useful, namely, the density. If I and I_o have the same significance as above, we may write

$$I = I_o \cdot 10^{-D}$$

D is called the density. And since

$$\text{Transmission} = \frac{I}{I_o}$$

$$D = -\log T$$

In measuring the amount of light absorption in solutions, reference is almost always made to thickness and concentration. It is much simpler to express absorption in terms that are directly proportional to the concentration

and thickness. For this reason density is used. This term is useful because the total density of a number of media is equal to the sum of their separate densities.

According to Beer's law, there should be a quantitative relationship between the amount of light absorbed and the concentration of the solution. The factor which expresses this quantitative relationship is usually designated as the absorption ratio (A), which is a constant for a particular substance and at a given wave-length. The determination of this constant depends not only on the spectrophotometric method of measurement, but also upon some other method which determines the concentration of the solution used. From the known concentration and density determined, the absorption ratio is calculated by:

$$C = A.D$$

The value of A for blood oxyhemoglobin at the peak of the beta absorption band from Kennedy's (2) work is 0.1165 when C is expressed in grams of hemoglobin per 100 cc. of sample. No value for A for muscle hemoglobin has been found in the literature.

HISTORICAL AND REVIEW OF LITERATURE

The study of the red pigments of blood and muscle is far from new. Hemoglobin of the blood and its relation to

body processes has been known for a long time. Muscle hemoglobin distinct from blood hemoglobin has been known relatively only a short while. There is an extraordinary difference in the volume of experimental work dealing with muscle hemoglobin and blood hemoglobin. Practically every laboratory of medicine, pathology, physiology, and related subjects has contributed one or more papers dealing with blood hemoglobin and its relation to body function. The papers dealing with muscle hemoglobin by contrast are hard to find and not easy to bring into harmony. Perhaps the chief reason for this difference in work done is because blood hemoglobin is very easy to obtain and analyze but muscle hemoglobin is hard to obtain free from blood hemoglobin and difficult to separate from the muscle proteins.

Since the first observations on the red pigment of muscle by Boerhaave (3) in 1775, the nature of this substance has been a debated question. Even modern methods and an increased knowledge of blood pigments have not entirely clarified the situation. There have been interesting fluctuations in the theories of physiologists as to the relation between the fixed muscle hemoglobin and the circulating blood hemoglobin. About 100 years ago it was thought that the muscle was impregnated with hemoglobin and if not all the hemoglobin was in the capillaries, there was a labile exchange between the muscle fibers and the blood hemoglobin,

an identity of red pigment material in the muscle and in the blood. About 50 or 60 years ago it was shown that the perfusion of muscle did not wash out all the red color, which seemed to be firmly fixed in the striated fibers. This indicated that there were two pigments.

Kuhne (4) considered it as ordinary hemoglobin and derived from blood vessels; others regard it as a special pigment actually formed within the muscle.

The visible absorption bands of oxyhemoglobin of blood first described by Hoppe-Seyler (5) have received considerable attention particularly as regards their position. Soret (6) was the first to recognize the great ultra-violet band of oxyhemoglobin, a band which he observed with a fluorescing eyepiece. MacMunn (7) described a respiratory pigment, which he found in muscles and other tissues. His experiments were repeated by Levy (8) who regarded the substance as being derived from hemoglobin. Hoppe-Seyler (9) fully supported Levy and refused to take into consideration the presence of 'myohematin' in invertebrates devoid of blood hemoglobin. Morner (10) as a result of his spectroscopic observations was the first to call attention to the fact that the alpha absorption band of muscle pigment did not occur at the same wave-length as for blood but was shifted 5 mμ toward the red end of the spectrum. The work of Morner was confirmed by Gunther (11) who suggested

"Myoglobin" as a name. Keilin (12) confirmed the position of the alpha band and called attention to the fact that the deviation is much larger than the one observed in hemoglobins from various species and therefore, should have a new name. Another pigment is reported by Keilin which he calls cytochrome. This pigment is an intracellular respiratory catalyst common to animals, bacteria, yeast and higher plants. Although present in all tissues, it shows marked absorption bands only in the reduced condition and does not interfere with a study of the oxyhemoglobins.

The belief that the two red pigments were distinct held sway for some time. However, it was shown that these hemoglobins had many characteristics in common. Hemin crystals can be formed from both and these crystals are identical. They show practically the same absorption curves and there is very little difference in color. Gunther suggests that the pigment fractions of muscle and blood hemoglobin are identical but that the globin fractions are different. As a result two well-defined schools of thought exist, those who claim this red pigment to be identical with the hemoglobin and those who do not.

The most recent observations are those of Kennedy and Whipple (13). These investigators used the spectrophotometer for their determinations and studied the color of solutions extracted from chopped red muscle by dilute

ammonia. They agree with previous observations as to the position of the bands but suggest that the cause of the deviation is due to fermentation taking place during extraction. They believed that there is no essential difference between the two hemoglobins. It has recently been shown by Ray and Paff (14) that the difference is not due to fermentation. Their method was based on determinations of the reflection curves from uninjured muscle immediately after its removal from the perfused animal. Later work of Kennedy and Whipple gives additional proof that muscle and blood hemoglobin are separate and distinct substances.

The idea that there is a labile exchange of hemoglobin between muscle and blood has been given up but some experimenters claim a fairly rapid fluctuation of muscle hemoglobin in response to hemoglobin fluctuations in the blood. This idea is discredited by the results obtained by Whipple (15) which show that the muscle hemoglobin level is very little reduced even after prolonged anemia. This indicates that anemia demands cannot rob the striated muscle of its hemoglobin. The principal factors affecting the muscle hemoglobin level are age and exercise according to Whipple (16). In young dogs he found very little muscle hemoglobin but the amount increased with age. The increase was more marked in the case of vigorous exercise. Exercise seems to be more potent than severe anemia. Lower muscle hemoglobin

values have been found in inactive house dogs with normal blood hemoglobin than in active dogs with one-third normal blood hemoglobin. Muscular paralysis is followed by a fairly rapid loss of muscle hemoglobin.

A further study of muscle hemoglobin by Whipple and Robscheit-Robbins (17) shows that muscle hemoglobin introduced intraperitoneally, intramuscularly or intravenously is promptly broken down and at least partly excreted as bile pigment in the urine of the dog. They also report (18) that blood hemoglobin introduced intraperitoneally or intravenously can be conserved by the dog having severe secondary anemia and 80 to 90 per cent of the introduced hemoglobin will appear in new formed red cells. The renal threshold for dog muscle hemoglobin is very low according to Manwell and Whipple (19), 11 to 15 milligrams per kilogram body weight. This amount is but 6 per cent of the value given for the renal threshold of dog blood hemoglobin. Very conclusive proof of the distinction between the two hemoglobins was presented by Hektoen, Robscheit-Robbins and Whipple (20). A specific precipitin reaction for dog muscle hemoglobin was prepared which differentiates it sharply from the blood hemoglobin of the dog. It is significant that the two substances behave differently on introduction into the blood stream. These experiments give added evidence that muscle hemoglobin is quite distinct biologically from blood

hemoglobin. The precipitin reaction also indicates that the difference may be in the globin fraction.

It has been reported that diet has no effect on the muscle hemoglobin level. However, data herein presented will indicate that diet may influence muscle hemoglobin formation.

THE SPECTROPHOTOMETER

The instrument used is a Bausch and Lomb spectrophotometer. It consists essentially of three units: (1) a means of illumination of the sample, (2) a rapid means of varying and comparing light intensities, (3) a spectrometer which divides the transmitted beam into its constituent colored components. This includes a spectrometer with an Abbe constant deviation prism, a photometer of the Martens type, and an adjustable upright specimen holder mounted on a suitable optical bench. The light source is a concentrated filament 250 watt lamp mounted at the end of the optical bench and completely housed except for a window of double ground glass. The photometer is mounted on the opposite end. Between the light source and the photometer is a vertical specimen holder of the colorimeter type which can be accurately controlled to one-tenth millimeter depth of solution by a vernier scale. Right angle prisms are used to alter the direction of the light beams and suitable lens systems serves to give parallel light through the cups and

plungers. The parallel beams after passage through the solutions are focused on the photometer aperatures, illuminating the field of the photometer. The photometer is of the polarization type in which the two halves of the field are polarized at right angles to each other by means of a Wollaston prism. Variation of intensity of the light between the two halves is made by the rotation of an analyser prism of the Glan-Thompson type, the rotation of which is read on a circle divided in degrees. The beams of light enter the photometer through two circular apertures of 5 mm. diameter which are separated by a distance of 40 mm. This separation is greater than usual and is made possible by the use of a bi-prism. On account of the slight polarization at the surfaces of the bi-prism, the match point is never exactly 45°. The method employed in calculating absorption is independent of the match point so that an exact setting at 45° is unnecessary.

The two beams from the photometer pass into the spectrometer and the spectra of the two are spread out in close juxtaposition before the eye. The prism of the spectrometer is mounted on a table which may be rotated by a micrometer screw which acts against a lug on the prism table and is operated by a knurled head outside the drum chamber. The distinctive feature of this instrument is that the telescope and collimator are fixed, and that the observer can pass

through the entire spectrum by rotating a drum head on which is read the wave-length of the portion of the spectrum in the middle of the field.

METHODS

Perfusion of Animal

The animal used for this work was a yearling Hereford steer in excellent condition weighing about six hundred and seventy pounds. The animal was thrown on its left side and shackled. An incision was made in the right side of the neck exposing the jugular vein and the carotid artery. A cannula was inserted into the carotid artery in such a manner that blood could be withdrawn at will. The animal was bled at a moderate rate from this artery until it had lost about one-fifth of its blood.

In order to prolong life and permit the heart to pump out a major portion of the circulating blood cells and also to dilute the blood, a sterile saline solution (0.85% Na Cl and 0.1% $Ca\ Cl_2 \cdot 6H_2O$) was injected by gravity into the vascular system through a T-tube inserted into the jugular vein. The fluid was injected at a rate approximately equal to that at which the blood was withdrawn from the artery. The heart's action ceased after this exchange of saline for blood had been continued for about ten minutes. In order to

wash from the vascular system as completely as possible the remaining traces of blood, the inflow cannula was changed to the carotid artery and the solution was allowed to flow into the arterial system. Perfusion was continued until the fluid flowing from the jugular vein was practically colorless. This required approximately twenty-one gallons of water. The animal was immediately opened, the paunch removed and the right hind quarter further perfused. The cannula was inserted into the femoral artery, the liquid escaping from the femoral vein. About nine gallons of saline solution were used. During the perfusion, the inflow of saline was occasionally interrupted for a number of seconds because Larson and Bell (21) have shown that intermittent perfusion is more efficient in washing blood cells out of the vascular system than a steady continuous flow. This first liquid that escaped from this quarter was quite red but after a few minutes the solution came through clear and colorless. Perfusion was stopped for a few seconds and then continued. This procedure was continued until only a very slight trace of color could be noticed. To aid further in adequately washing out the smaller vessels, the hind quarter was thoroughly massaged and flexed during perfusion.

The carcass was allowed to hang in the cooler at about 32°F. for two days, after which it was cut up and the desired muscles removed. All visible fat was removed, after

which the muscle was ground and one-hundred gram portions put into small sample bottles. These were kept in a refrigerating room at an average temperature of -12°F. until wanted.

Preparation of Muscle Hemoglobin Solution

Two one-hundred gram portions of meat were removed from the refrigerating room and allowed to thaw out in an electric refrigerator until the meat could be cut out with little difficulty. This was placed with 250 cc. of ice water into a previously cooled pebble mill (one quart size). The mill was run for one and one-half hours, keeping the jar cold in warm weather with an ice bag or kept out doors in near freezing weather. At the end of this time, the meat was thoroughly macerated and the muscle hemoglobin extracted. This mixture was centrifuged and then filtered through a linen cloth to remove the solid fat. To this solution 0.1 gram of solid sodium carbonate was added to each 100 cc. of solution. This solution was very turbid. To clarify the solution and remove the muscle proteins, aluminum hydroxide was added and shaken vigorously for ten minutes after which it was filtered in the refrigerator. After this treatment, the solution was much clearer but showed a decided Tyndall effect, so it was again treated with aluminum hydroxide.

The resulting solution was bright red in color and quite free from colloidal material. Only a very small amount of the muscle hemoglobin was removed by the aluminum hydroxide.

Preparation of Blood Hemoglobin Solution

Blood was obtained from the jugular vein of a cow with a large hypodermic needle. The blood was caught in a test tube containing a small amount of solid sodium citrate. To separate the blood hemoglobin from the other soluble blood proteins and the yellow pigment of the plasma, the blood was centrifuged, the plasma taken off and the red cells were washed and centrifuged with saline solution (0.85% Na Cl and 0.1% $Ca\ Cl_2 . 6H_2O$) several times until the supernatant liquid was clear and colorless. The red cells were laked with 0.1 per cent sodium carbonate solution and made up to volume. Aluminum hydroxide was added and shaken vigorously for ten minutes. This gave a solution that was clear and free from colloidal material. Marshall and Welker (22) have shown that aluminum hydroxide removes colloidal material quantitatively and that blood oxyhemoglobin is the only protein of blood that is not removed by it.

However, quite a noticeable amount of blood oxyhemoglobin is removed by aluminum hydroxide. The precipitate retained on the filter paper is highly colored with this pigment.

They report also that other proteins, including egg albumin, gelatin, casein, nucleoprotein, primary and secondary proteoses are completely removed, the biuret test giving negative results.

Preparation of Solutions for Colorimetric and Spectrographic Analysis

The acid hematin solutions for colorimetric comparison were prepared by adding an equal volume of 0.2 N H Cl to the 0.1 per cent sodium carbonate solution of the hemoglobins and allowing to stand for one hour. Newcomer (23) reports that the acid hematin color does not reach its full density until twenty-four hours have passed but that it develops rather rapidly at first and has reached 99 per cent of its full intensity at the end of 40 minutes.

For the spectrographic analysis of the acid hematin and the globin fractions separately, the solutions were made by adding one-half as much glacial acetic acid and an equal volume of ether to the 0.1 per cent sodium carbonate solutions. This separated into two layers and the fractions were drawn off from a separatory funnel. The acid hematin was soluble in the ether layer.

Spectrophotometric Measurements

The spectrometer was placed before a mercury arc light

and the ocular slit adjusted so that the reading shown by the wave-length drum corresponded to the accepted values for the mercury lines. The error of the wave-length drum was not more than 0.3m μ for any line. This setting was also checked using sodium and lithium flames. The collimator slit is controlled by a micrometer screw with a head reading to 0.1 mm. In the spectrophotometric analysis of the muscle and blood hemoglobin solutions, the collimator slit never exceeded 0.1 mm. except in regions below 520 m μ . According to Fechner's law, the minimal difference which can be distinguished in the intensity of adjacent fields, is proportional to the total intensity and is about 1 to 2 per cent under ideal conditions. Therefore, the collimator slit was varied so that the observation field was always at minimal brightness in order to be able to detect slight differences in intensity. The eyepiece of the spectrophotometer was kept narrow so as to limit the region of the spectrum observed to approximately 3 m μ .

One cell was filled with the solution to be observed. A similar cell was filled with the solvent to correct for any light scattering or absorption due to the cell and solvent. The spectrophotometer was adjusted and the two solutions were placed at the desired depth in the paths of the two light beams. The wave-length drum was set for the portion of the spectrum to be observed and the analysing prism

of the photometer was rotated until a match in the comparison field was obtained. The total intensity of the field was adjusted by the collimator slit. Record was made of the degrees on the scale. The wave-length was changed and similar readings taken until the range of the visible spectrum had been covered. The photometer was reversed and the entire spectrum read as before.

The usual formula given for a Martens type photometer is:

$$T = \frac{I}{I_o} = \tan^2 \theta$$

where I and I_o have the same significance as already stated and θ the angle of rotation of the analysing nicol. The above formula assumes a match point of 45°, and also assumes that the substance is optically inactive. One of the advantages of the Martens type of photometer is that the effects of any polarization due to the specimen and of any deviation of the match point from 45° is completely compensated by reversing the photometer. If θ_1 is the scale reading of the analysing nicol for match when the sample is covering the half of the field which is extinguished for $\theta = 0^\circ$, θ_1 will vary from 45° to 90°. If θ_2 is the scale reading of the analysing nicol for match when the sample is covering the half of the field which is extinguished by $\theta = 90^\circ$, θ_2 will

vary from 0° to 45°. Then,

$$T = \frac{\tan \theta_2}{\tan \theta_1} = \tan \theta_2 \times \cot \theta_1$$

or $$D = \log \tan \theta_1 + \log \cot \theta_2$$

EXPERIMENTAL OBSERVATIONS

When the round was cut, it was found to be quite moist and leaked slowly. This exudate, which was very pale red in color, proved to be colored almost wholly by muscle hemoglobin. An examination in the spectrophotometer showed the absorption bands decidedly shifted as for muscle hemoglobin. The organs of the animal were all well perfused. Very little liquid was left in any of them and this liquid was either very pale red or colorless.

Spectrophotometric determinations of the density for the entire range of the visible spectrum were made. Fig. 1 A and C shows the absorption curves for blood and muscle hemoglobin in 0.1 per cent sodium carbonate solution when the light absorbed by them is practically the same. Curve B is the absorption for a blood hemoglobin solution, the concentration of which is equal to that of the muscle hemoglobin solution used in obtaining curve C. The curves are very similar except that the curve for muscle hemoglobin is shifted toward the red end. Also, less light is absorbed by

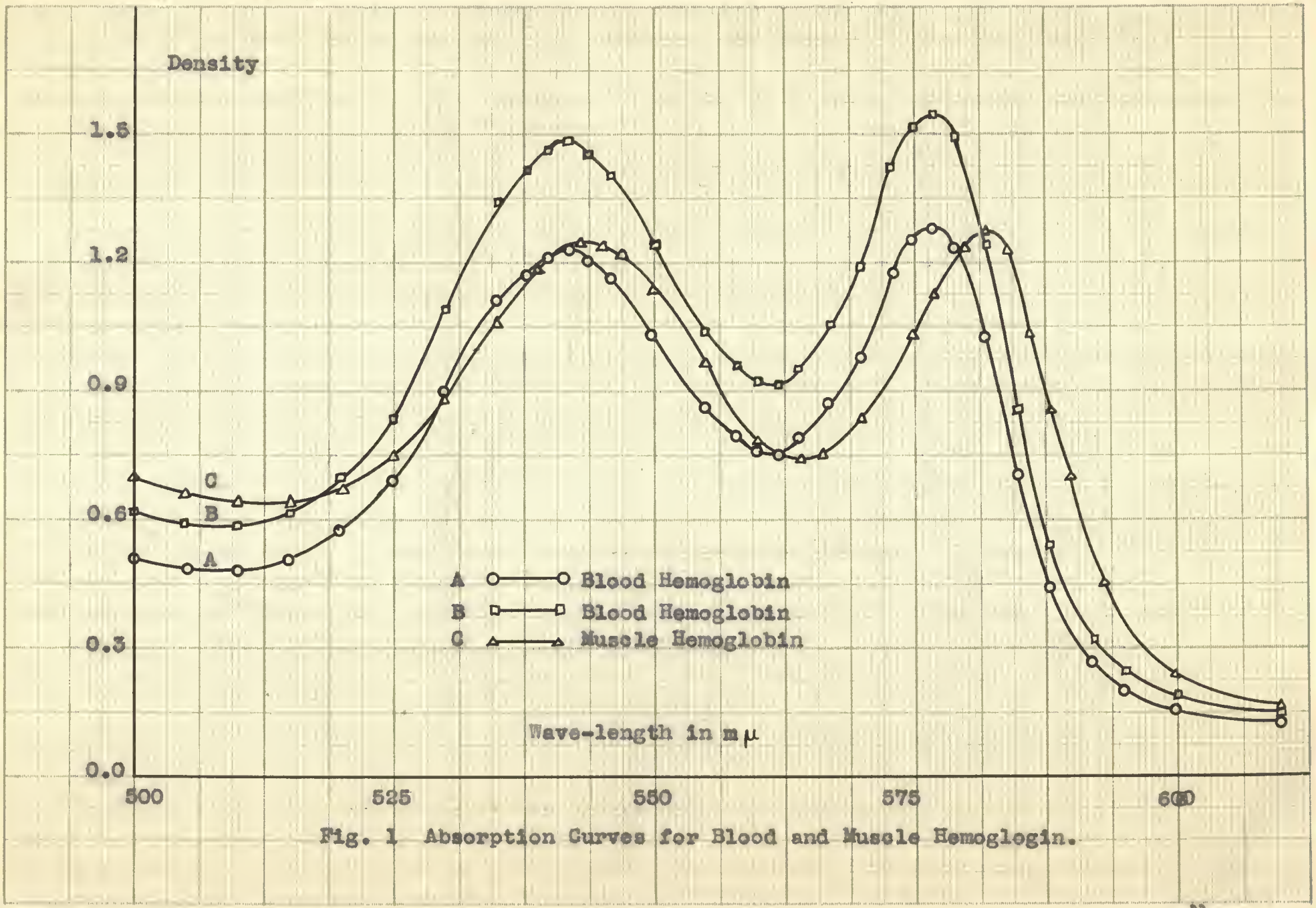

Fig. 1 Absorption Curves for Blood and Muscle Hemoglogin.

blood hemoglobin at 510 mμ but neither absorbs much red light. Obviously there are three important points in these absorption curves; the maximum in the green, the maximum in the yellow and the minimum between these maxima. These may be taken as reference points at which optical constants can be determined with greatest accuracy.

The points of maximum absorption for blood hemoglobin were found to occur at 577 mμ, and 542 mμ, the minimum at 562 mμ as compared to the corresponding values for muscle hemoglobin 582 mμ, 543 mμ and 564 mμ. At least fifteen solutions of each hemoglobin were used in determining the position of these points.

It was found that muscle and blood hemoglobin solutions could not be compared directly in the colorimeter because the two colors differ in value. Therefore, solutions of equal concentration were made by comparing the acid hematin derivatives in N/10 H Cl. There is no noticeable difference in the color of the two acid hematin derivatives and comparison could be made quite accurately. The two hemoglobin solutions of equivalent concentration made up by this method did not show the same power of absorption.

The ratio of the densities of the maximum in the green to the minimum has been used to identify blood oxyhemoglobin but no mention of ratios including the maximum in the yellow was found. Table I gives the ratios of the densities

Table I. Ratios of Densities for Blood Hemoglobin

Sample	D542/D562	D577/D542	D577/D562	D577/D582
1	1.638	1.049	1.650	1.211
2	1.606	1.066	1.712	1.230
3	1.614	1.059	1.710	1.237
4	1.646	1.055	1.720	1.224
5	1.656	1.038	1.709	1.241
Average	1.632	1.053	1.700	1.230
Mean Diff.	0.017	0.008	0.020	0.009

of these three points already mentioned and also the ratio of the maximum in the yellow to the point of the maximum for muscle hemoglobin. It was found that all of these ratios were quite constant. Also the ratio of the two maxima is more constant than that used to identify this pigment. The ratio of the densities of the maximum in the green to the minimum is given by Kennedy (2) as 1.63 which agrees very well with the results obtained. Ratios of the corresponding points for muscle hemoglobin are given in Table II. It will be noticed that the values given here are very little different than the values for blood hemoglobin at corresponding points except the ratio D(577)/D(582). Since there is a difference of 5 mμ between the points of maximum absorption of yellow light, and also considerable difference between the amount of light absorbed by the two at equal concen-

Table II. Ratios of Densities for Muscle Hemoglobin

Sample	D543/D564	D582/D543	D582/D564	D577/D582
1	1.602	1.011	1.620	0.882
2	1.697	1.043	1.770	0.874
3	1.656	1.005	1.635	0.888
4	1.675	1.014	1.697	0.894
5	1.609	1.023	1.709	0.882
Average	1.648	1.019	1.686	0.884
Mean Diff.	0.034	0.011	0.047	0.006

tration, it was decided to use this ratio in an attempt to determine the relative percentage of the two hemoglobins in a mixture.

From solutions of equal concentration of blood and muscle hemoglobin in 0.1 per cent sodium carbonate, mixtures were made at intervals of 20 per cent from pure blood hemoglobin to pure muscle hemoglobin. The optical density of the solution at 577 mμ and 582 mμ was determined and the ratio calculated. The results of five runs are given in Table III. The ratio for each mixture was found to be quite constant. Plotting the average of these densities against per cent hemoglobin gives practically a straight line as shown in Figure 2. For pure blood hemoglobin, this ratio was found to be 1.230. If whole blood is diluted with 0.1 per cent sodium carbonate solution, this ratio is found to be

Table III. Ratio D577/D542 for Mixtures

	100% Blood	80%: 20%	60%: 40%	40%: 60%	20%: 80%	100% Muscle
1	1.224	1.138	1.085	1.052	0.945	0.894
2	1.237	1.163	1.103	1.031	0.942	0.888
3	1.230	1.169	1.096	1.011	0.946	0.874
4	1.211	1.138	1.103	1.028	0.943	0.882
5	1.241	1.176	1.122	1.039	0.963	0.888
Average	1.230	1.157	1.096	1.030	0.944	0.884
Mean Diff.	0.009	0.015	0.010	0.011	0.005	0.006

1.072, probably due to a pigment in the plasma. To determine the relative per cent of muscle and blood hemoglobin in a solution, it is only necessary to determine this ratio and the per cent read from the curve. The per cent of each in a sample may be obtained by a determination of the acid hematin or by determining the density at 542 m μ and using the absorption ratios of the two hemoglobins.

In the preliminary work a number of muscle extracts were obtained from the Chemistry Analytical Laboratory. These extracts were the supernatant liquid from 100 grams of meat ground with 300 cc. water in a pebble mill. To these solutions 0.1 gram of solid sodium carbonate was added and then filtered until clear, adding a little calcium chloride if necessary to flocculate colloidal material. The ratio of the densities at 577 m μ and 542 m μ was determined for

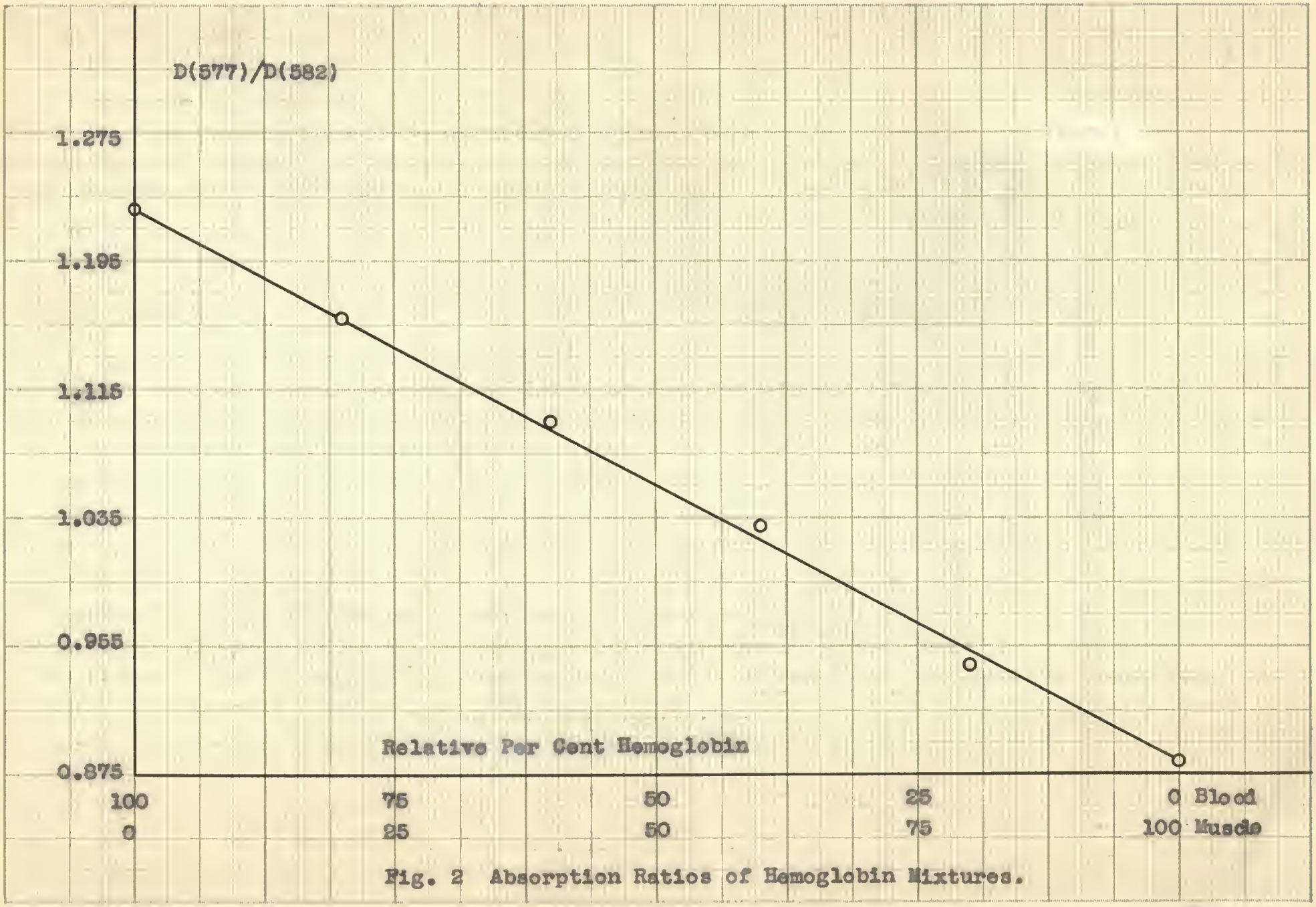

Fig. 2 Absorption Ratios of Hemoglobin Mixtures.

each solution and the relative per cent of each hemoglobin read from Figure 2. The results are given in Table IV and also the percentage of each in the meat samples.

Table IV. Muscle and Blood Hemoglobin Content of the Rib Eye Muscle of Beef Animals

Animal No.	Relative % Hemoglobin Muscle Hb	Blood Hb	Per cent of Meat Muscle Hb	Blood Hb
		Lot I		
87	93.0	7.0	0.355	0.027
202	100.0	0.0	0.324	0.000
224	84.0	16.0	0.349	0.066
263	81.0	19.0	0.302	0.071
Average	89.5	10.5	0.332	0.041
		Lot II		
96	90.5	9.5	0.360	0.038
294	91.0	9.0	0.391	0.039
47	95.5	4.5	0.379	0.018
281	88.0	12.0	0.419	0.057
Average	91.25	8.75	0.387	0.038
		Lot III		
I_s	95.5	4.5	0.439	0.021
I_h	98.0	2.0	0.469	0.010
Average	96.75	3.25	0.454	0.015

Ration:

Lot I. 13.56# corn, 2# alfalfa hay and cottonseed meal daily.

Lot II. 12.55# corn, pasture and cottonseed meal daily.

Lot III. Pasture.

The percentages in the meat were determined as follows:

$$C = A.D$$

at equal concentrations, we have:

$$A_m . D_m = A_b . D_b$$

$$A_m = \frac{A_b . D_b}{D_m}$$

Using the densities determined at 542 mμ and the absorption ratio for blood, 0.1165 g. per 100 cc. given by Kennedy (2), the absorption ratio for muscle hemoglobin was found to be 0.1410 g. per 100 cc. Then since the total density is equal to the sum of the separate densities, we may write,

$$\frac{C_m}{0.1410} + \frac{C_b}{0.1165} = D542$$

$\frac{C_m}{C_b} = X$ or $C_m = X.C_b$, X is calculated from the relative percentages.

Then,

$$\frac{XC_b}{0.1410} + \frac{C_b}{0.1165} = D542$$

From this C_b can be calculated and C_m is found by substituting C_b in either of the other equations. These values must be multiplied by four to obtain the per cent hemoglobin

in the meat, since the hemoglobins in 100 grams of meat were distributed through 400 grams of material. Table IV shows the muscle and blood hemoglobin content of the rib eye muscle of beef animals. The relative percentages were obtained by the use of Figure 2 and the actual percentages calculated by the method just outlined.

Although there was no noticeable difference in color of the acid hematin solutions and no difference was noted in the visible spectra, additional proof was desired as to the identity of the acid hematins and difference of the globin fractions. Solutions of the two fractions were made as outlined in the methods. In this work, light from an arc of impregnated carbons was passed through a quartz cell 9 mm. thick which contained the material to be examined. A Bausch and Lomb quartz spectrograph was used, the spectra being photographed on Eastman panchromatic plates.

In Figure 3 the absorption spectra of muscle and blood hemoglobins are shown. The time of exposure is too long to show the alpha and beta absorption bands but both hemoglobins show a wide absorption at the beginning of the ultra-violet. The width of this band depends on the concentration of the solution and the time of exposure. There is a decided difference in absorption below 295 mμ . All of the light is absorbed by the muscle extract while blood hemoglobin allows transmission as far as 246 mμ . This

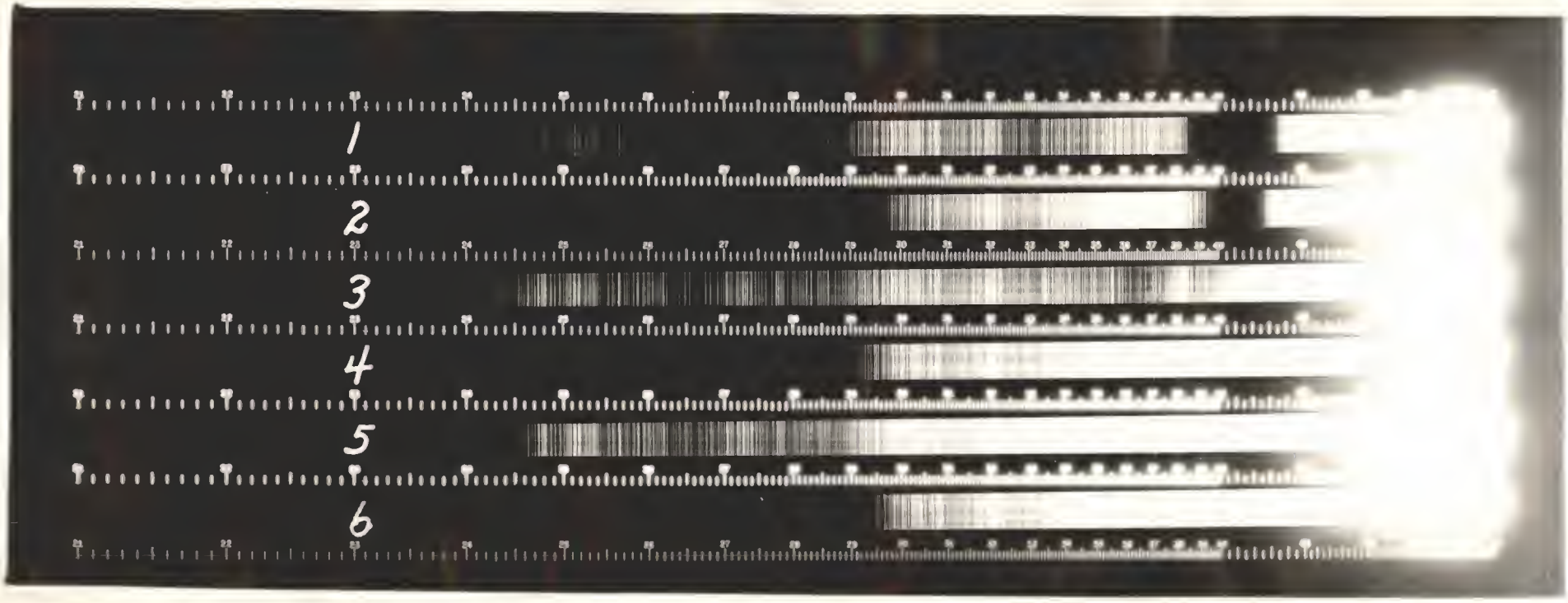

Fig. 3 Absorption Spectra of Blood and Muscle Hemoglobins and Derivatives.

1. Blood Hemoglobin in 0.1 per cent sodium carbonate solution
2. Muscle Hemoglobin in 0.1 per cent sodium carbonate solution
3. Blood Acid Hematin in N/10 H Cl.
4. Muscle Acid Hematin in N/10 H Cl.
5. Globin Fraction of Blood Hemoglobin
6. Globin Fraction of Muscle Hemoglobin

same difference in absorption is shown in the spectra of the acid hematin derivatives in N/10 H Cl. Figure 4 shows that there is the same marked difference in the absorption spectra of the globin fractions but very little difference in the absorption spectra of the acid hematin fractions. This difference may be due to the globin, some of which may be soluble in the ether fraction.

In Figure 5 the absorption spectra of the two hemoglobins in the visible, taken with the camera attachment for the spectrophotometer are shown. Also the absorption spectra of mixtures are given which show a characteristic shift toward the red portion of the spectrum each time the concentration of muscle hemoglobin is increased. At the bottom, the absorption spectrum of blood hemoglobin is shown with that of muscle hemoglobin and the displacement of absorption bands is quite noticeable.

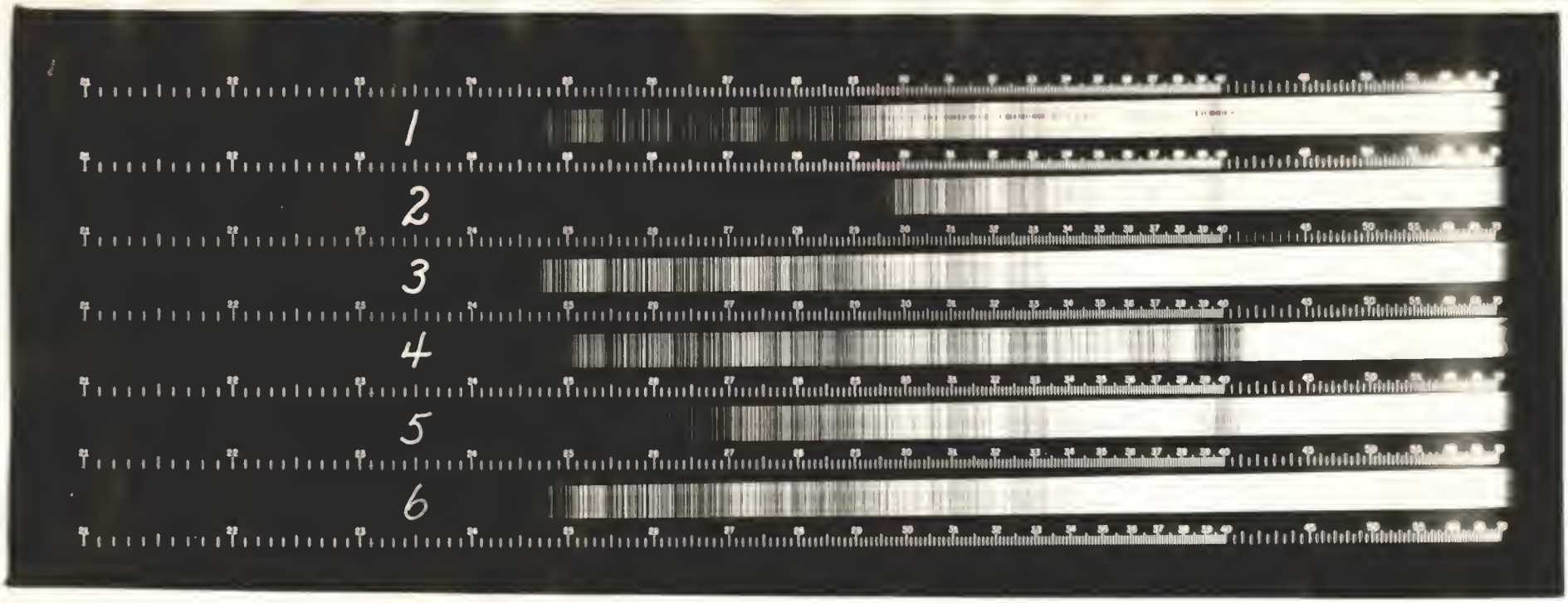

Fig. 4 Absorption Spectra of Globin and Acid Hematin Fractions of Blood and Muscle Hemoglobins.

1. Globin Fraction of Blood Hemoglobin
2. Globin Fraction of Muscle Hemoglobin
3. Solvent for globin
4. Acid Hematin Fraction of Blood Hemoglobin
5. Acid Hematin Fraction of Muscle Hemoglobin
6. Solvent for Acid Hematin

CA
CR

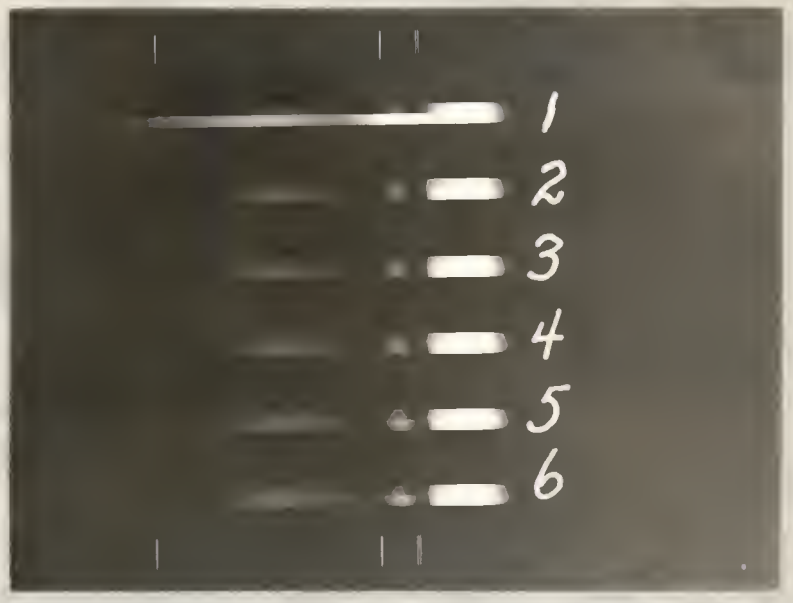

Fig. 5 Absorption Spectra of Mixtures of Blood and Muscle Hemoglobin in 0.1 per cent Sodium Carbonate Solution. For comparison, the Spectrum of Blood Hemoglobin was photographed above each mixture.

1. Blood and 0.1 per cent Sodium Carbonate Solution
2. Blood and 80% Blood-20% Muscle Mixture
3. Blood and 60% Blood-40% Muscle Mixture
4. Blood and 40% Blood-60% Muscle Mixture
5. Blood and 20% Blood-80% Muscle Mixture
6. Blood and Muscle Hemoglobins

The mercury arc spectrum is given above and below for comparison.

DISCUSSION

On theoretical grounds, it is not expected that all of the blood can be washed out of the muscle with a perfusing solution since the solution is much thinner than the blood and there is less friction on the walls of the vessels. As soon as a passage is opened, resistance along this route will be much less than through capillaries containing blood and the fluid will take the path of least resistance. However, using the method of simultaneous bleeding and perfusion together with massage and intermittent perfusion of the particular muscles desired, it is believed that the muscle hemoglobin prepared is quite free from blood hemoglobin. Also it was observed that aluminum hydroxide shaken with the two hemoglobins removes more of the blood hemoglobin than of the muscle hemoglobin. A solution containing equal concentrations of the two was shaken with aluminum hydroxide and filtered. The relative percentage of muscle hemoglobin in the filtered solution was about 90 per cent.

The absorption curves are found to be very similar but the muscle hemoglobin curve is shifted toward the red portion of the spectrum. The points of maximum absorption determined differ about 2 m μ from the results of other investigators but the distance between the points is the same.

Although no work was found indicating that diet influences the level of muscle hemoglobin, an examination of Table IV would lead one to believe that it does. The Lot I animals were given corn, cottonseed meal and alfalfa hay in dry lot, animals of Lot II were kept on pasture supplemented by corn and cottonseed meal, while the Lot III animals were wholly on pasture. The difference between the hemoglobin levels of the animals of Lots I and II could be explained by assuming that the animals on pasture had more exercise. This explanation would not be sufficient to show why the Lot III animals have such a high hemoglobin level so it would seem that exercise is not the all important factor and that diet does have an influence on the muscle hemoglobin level. Also the table shows that there was less residual blood left in the meat of the pasture animals. Muscle hemoglobin is much more stable than blood hemoglobin. A change in the muscle hemoglobin level is not readily produced by change of diet. A long time is necessary to produce much change, while nutritional anemia can be quite readily produced.

SUMMARY

Solutions of muscle hemoglobin free from blood hemoglobin were prepared and examined by the spectrophotometer. These solutions gave absorption curves very similar to the

curves obtained from blood hemoglobin but they are displaced toward the red portion of the spectrum. These absorption curves are shown.

Equal concentrations of the two hemoglobins do not have equal power of light absorption. The two hemoglobins cannot be matched directly since the colors of the solutions do not have the same value, but their acid hematin derivatives have been shown to be identical.

The ratio of the densities of the maximum absorption in the yellow to the maximum in the green, the ratios of both maxima to the minimum between them and the ratio of the densities at 577 mμ to 582 mμ were found to be constant.

The points of maximum absorption for blood oxyhemoglobin were found to be at 542 mμ and 577 mμ, the minimum at 562 mμ as compared to 543 mμ, 582 mμ and 564 mμ, the corresponding points for muscle oxyhemoglobin.

A spectrographic study shows a very marked difference in the absorption spectra of the two hemoglobins and the same difference in the absorption spectra of the globin fractions. Very little difference was shown between the acid hematin fractions indicating the difference is in the protein part of the molecule.

A method for determining the relative percentages of blood and muscle hemoglobin in the same solution based on the ratio of the optical densities at 577 mμ and 582 mμ is presented.

The results here submitted indicate that diet influences the muscle hemoglobin level. Animals on pasture have a higher level than dry lot fed animals and the difference cannot all be explained by the assumption of the pasture animals having more exercise.

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