CCLII. SOME OBSERVATIONS ON THE ESTIMATION OF MUSCLE HAEMOGLOBIN.

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NUMEROUS observations in the past decade, culminating in the isolation of the crystalline material and the study of its properties by Theorell [1932; 1934, 1, 2, 3, 4] have established the identity of muscle haemoglobin. Attempts to estimate it, however, have for the most part been concerned with it less as a chemical identity than as that haemoglobin which remains behind after perfusing the tissue apparently free of blood. The most notable of these is that of Whipple [1926]. Though his estimations show consistency, it is not certain that they represent the actual concentrations of muscle haemoglobin. There is the possibility that some haemoglobin has been lost in the perfusion of the tissue and uncertainty both as to whether there is an equal distribution of muscle haemoglobin between the tissue and the extracting fluid and as to the actual volume of the tissue. Further there is the possibility of other acid haematin compounds being extracted from the cell and introducing error into the subsequent acid haematin determination. Finally the conclusions drawn from the colorimetric comparison of the carboxy-compounds may be incorrect, for Theorell [1934, 2] has since shown that the light absorptions by the carboxy-haemoglobins of muscle and blood are different.

In this paper is reported an attempt to develop a method for a more exact estimation by extracting the whole of the haemoglobin from the unperfused tissue and by determining the relative concentrations of muscle and blood haemoglobin in the extract from the mean position of the α -band of the oxy-haemoglobin compounds as observed with the Hartridge reversion spectroscope and the determination of the total concentration of haemoglobin spectrophotometrically. Some observations on Whipple's [1926] method of extraction, the outcome of a comparison of it with the method developed, are also reported.

Shenk *et al.* [1934] describe a method somewhat similar in principle. These authors are to be commended for their heroic effort in perfusing an ox. There exist, however, discrepancies between the spectrophotometric constants obtained with their extract of the perfused tissue and those obtained by Theorell [1934, 2] with the crystalline horse muscle haemoglobin. Though these may be due to the difference in the source of the haemoglobin, it has been the experience of the author that it is extremely difficult to perfuse a tissue entirely free of blood haemoglobin. Further, the same uncertainty as to the distribution of the haemoglobin between the tissue and the extracting fluid *etc.*, exists as in Whipple's [1926] method.

Extraction of haemoglobin. For the purpose of complete extraction, it was necessary that the muscle should be in as fine a state of division as possible. To this end, minced muscle ground with sand was employed. The tissue juices

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slowly separate from minced muscle and sink towards the bottom of the mass. The tissue sample should therefore be weighed out immediately after mincing.

As Whipple [1926] found, water extracts the haemoglobin incompletely (see Table I). When the tissue was repeatedly extracted with *M*/15 phosphate buffer for periods of 3 hours, the fourth and subsequent extracts contained very little haemoglobin indeed, and after the fourth extraction no haemoglobin could be detected in the extracted tissue using the microspectroscope, the tissue being reduced to show strong cytochrome bands and then reoxidised until these had disappeared. On comparison of the acid haematin compounds of combined extracts 4 and 5 with those of combined extracts 1, 2 and 3 from a sheep heart muscle extraction, the former was found to be 2.4% of the latter on one occasion and 4.5% on another. It is considered that not more than 5% of the total haemoglobin is left behind after repeating the extraction twice, if not more than 6 g. of tissue be used and the tissue be thoroughly ground with sand. The final solution becomes very dilute if the extraction is repeated further.

Whilst alkaline phosphate buffer will extract the haemoglobin completely, difficulty arises in the filtering, the filter tending to clog. In slightly acid phosphate buffer the extracts filter clear quite readily when shaken with kieselguhr and less readily, in fact rather slowly, with aluminium hydroxide. In acid solution, however, haemoglobin is absorbed by kieselguhr and aluminium hydroxide. It is readily washed off with alkaline solutions but, unfortunately, such solutions deflocculate the kieselguhr so that the washings are cloudy. These washings, however, when shaken with aluminium hydroxide, filter clear. Using a combination of kieselguhr and aluminium hydroxide, a crystal-clear filtrate may be obtained in a much shorter time than by using aluminium hydroxide alone.

That the adsorbed haemoglobin may be almost completely washed off was shown by shaking aliquots of a clear solution with further kieselguhr and aluminium hydroxide. With aluminium hydroxide the recoveries were 95 and 96% in two cases tried. With kieselguhr, the apparent recoveries were 104 and 105% owing to the faint cloudiness produced on washing with an alkaline solution. In both cases the amount of haemoglobin present was much less than in an ordinary determination.

For quantitative extraction, the following procedure, taking into account these various considerations, has been found a convenient one: 5–6 g. of well-minced muscle tissue are ground with an equal volume of sand. The well-ground mass is washed into a 25 ml. centrifuge-tube with 12–14 ml. *M*/15 phosphate buffer, about p_H 6.5, and shaken gently for 3 hours. It is then centrifuged for 5 minutes at about 3000 r.p.m. and the supernatant liquor poured off and stored in the refrigerator. Another 12–14 ml. phosphate buffer is added, the tissue thoroughly stirred up with it and again extracted for 3 hours, after which the fluid is centrifuged off as before. This process is repeated a second time. The combined extracts are then centrifuged for 45 minutes and the supernatant liquor is poured off, shaken up with kieselguhr and filtered by suction, the filtrate being put through a second time after which it is usually crystal-clear, but, if not, it is put through still a third time. The residue on the filter-paper is washed with a few ml. of 0.1% ammonium hydroxide till the washings which are collected separately are quite colourless. These washings are shaken with aluminium hydroxide and filtered afresh under gentle suction, this residue on the filter-paper also being washed with 0.1% ammonium hydroxide. The extracts and washings are made up to 60 ml.

Determination of the concentrations of muscle and blood haemoglobin relative to one another in a mixture of the two. It having been established that the mean position of the α -band of muscle oxyhaemoglobin is nearer the red end of the spectrum than that of blood oxyhaemoglobin, the mean position of the band in mixtures of the two oxyhaemoglobins suggested itself as a possible means of determining the relative concentrations of the haemoglobins in the mixtures. The nature of the relation between the mean position of the α -band and the relative concentrations of the haemoglobins had to be determined.

Crystalline muscle haemoglobin of the horse was prepared after the method of Theorell [1932]. (It was redialysed twice, the crystalline mass being well washed each time with saturated ammonium sulphate solution.) It was taken up in phosphate buffer at p_H 6.7. On the grounds of the observations of Roche [1932], the concentrations of the solutions relative to one another was obtained by colorimetric comparison of the acid haematins. To 7 ml. of solution 1 ml. of approximately $NHCl$ was added and after 3 hours the acid haematins were compared colorimetrically. An excellent colour match was obtained. After storage of the haemoglobin solutions for 1 month in the refrigerator, the relative concentrations were still unaltered. Each of these solutions and various mixtures of the two were reduced with Stokes's reagent (alkaline ferrous tartrate) and reoxidised and the mean position of the α -band determined with the Hartridge reversion spectroscope immediately. All readings were made at 21° and at the same concentration. They are plotted in Fig. 1.

Fig. 1. The relation between the % muscle oxyhaemoglobin in a mixture of muscle and blood oxyhaemoglobins and the mean position of the α -band.

The observed position of the α -band of crystalline muscle oxyhaemoglobin of the horse is 5808 Å. , 5 Å. nearer the red than was found by Roche [1932]. It is possible that Roche's solutions may have contained a little blood oxyhaemoglobin, but it is to be remarked that the observed value of the blood oxyhaemoglobin of the horse, $5774\text{--}5\text{ Å.}$, is 5 Å. nearer the red than is reported by Barcroft [1928]. By comparison with the observations of Theorell [1934, 2], using the spectrophotometer, it would appear that the mean position of the α -band differs somewhat from the position of its maximum absorption. As is evident from Fig. 1, this mean position of the α -band changed uniformly throughout the

interval of 33–4 Å. as the relative concentrations of the oxyhaemoglobins to one another were changed.

It is, therefore, simple to obtain the relative concentrations of the haemoglobins in extracts of horse muscle tissue. They need only be brought to a temperature of about 20–21°, reduced with Stokes's reagent and reoxidised and the mean wave-length of the α -band determined with the Hartridge reversion spectroscope immediately. The relative concentrations can then be read off from Fig. 1, the concentration of the solutions from which Fig. 1 was derived having been approximately the same as those found in extracts. The adjustment of the temperature is necessary in view of the observations of Hartridge [1923] that the mean position of the α -band of oxyhaemoglobin changes with the temperature. A further necessary precaution is gentle treatment of the extract during reduction and reoxidation, as vigorous shaking produces cloudiness in the solution, a phenomenon reported by Douglas *et al.* [1912] for dilute haemoglobin solutions.

There is no reason to believe that a different relation will exist with the blood and muscle oxyhaemoglobins of other animals. Once then the mean positions of the α -bands of these blood and muscle oxyhaemoglobins have been established, their concentrations relative to one another in tissue extracts can be determined.

Spectrophotometric determination of the concentration of the total haemoglobins in extracts of muscle. For this purpose the oxy-compound is unsuitable owing to the ease with which muscle oxyhaemoglobin changes into muscle methaemoglobin. The carbon monoxide compound of muscle haemoglobin can be used but, as Theorell [1934, 2] showed, it exhibits a relatively slight difference in its affinities for oxygen and carbon monoxide, so that the absence of oxygen must be ensured. As mentioned above, shaking, or for that matter any treatment which leads to the formation of bubbles, causes a precipitation of material. This, undesirable in itself, as being a direct source of spectrophotometric error, may to some extent cause loss of haemoglobin by denaturation.

The following procedure was applied: 10–15 ml. of the tissue extract were transferred to a large vacuum tube, sodium hydrosulphite added in certain excess of the amount necessary to remove all oxygen and the air replaced by coal gas or carbon monoxide. The vacuum tube was then gently rotated for a few minutes, after which a 1 ml. cell was completely filled with the carboxyhaemoglobin solution and quickly covered with a cover glass so that no air bubbles were retained. The spectrophotometer readings were made immediately at 6300, 5600 and 5400 Å.

The concentration of haemoglobin in the solution was calculated with the help of the formula

$$\epsilon = \frac{1}{cd} \log I_0/I,$$

where ϵ is the specific extinction coefficient, c is g. of haemoglobin per litre of solution, d is the thickness of the spectrophotometric cell and I_0/I is the relation of incident to transmitted light.

The only outstanding figures for the specific extinction coefficients of muscle and blood carboxyhaemoglobins are those of the haemoglobins of the horse. At 5600 and 5400 Å. the specific extinction coefficients of crystalline horse muscle carboxyhaemoglobin are 0.55 and 0.71 respectively from the figures of Theorell [1934, 2] and of crystalline blood carboxyhaemoglobin 0.78 and 0.87 respectively from the figures of Haurowitz [1934]. It was assumed that these spectrophotometric constants of the carboxyhaemoglobins of the horse apply to the haemoglobins of the other domestic animals and that the specific extinction

coefficient of a mixture of muscle and blood carboxyhaemoglobins will vary between these values in direct linear relation with their relative concentrations in the mixture.

Having obtained the relative concentrations by means of the Hartridge reversion spectroscope, the specific extinction coefficient of the mixture could be calculated. The concentration of haemoglobin in the extract was then

$$\frac{\text{observed log } I_0/I}{10 \cdot \epsilon \text{ mixture} \cdot d} \text{ g./100 ml.,}$$

and in the tissue

$$\frac{\text{observed log } I_0/I \cdot v}{10 \cdot \epsilon \text{ mixture} \cdot d \cdot w} \text{ g./100 ml.,}$$

where v is the volume in ml. of the tissue extract and w the weight of the tissue sample in g.

The extinction at 6300 Å. was always appreciable, though according to Theorell [1934, 2] and Haurowitz [1934] it is negligible. In two or three cases where it was investigated, it was found that the absorption was general throughout the red, there being a slight gradual increase from 6800 to 6000 Å. As usually some faint opalescence was still present in the solution, it is considered that this absorption might have been due to it. In calculations, therefore, the values for $\log I_0/I$ used were always the differences between $\log I_0/I$ at 5600 or 5400 Å. and at 6300 Å., this being considered to be a nearer approximation to the true value. Appreciable absorption was found by Whipple [1926] in this region.

Roche [1932] having shown that the haematin of blood haemoglobin is identical with that of muscle haemoglobin, the experience gained in the course of the work indicates that the comparison of the acid haematin of the tissue extract with that of a blood standard may possess advantages over the spectrophotometric estimation. Excellent colour matches have been obtained between acid haematin solutions prepared from a solution of blood haemoglobin and from tissue extracts, indicating that neither the different globin nor the protein content of the extract affects the dispersion of the acid haematin appreciably. As mentioned in the introduction, however, there still remains the uncertainty as to the amount of haematin other than that of haemoglobin origin which is extracted from the tissue.

In Table I are presented the results of determinations made during the course of the work. The ox, sheep and pig hearts were obtained over a period of some months from animals at the slaughter house shortly after they were killed; the "shin beef" came from a dressed carcass; the dog heart and leg came from the one animal shortly after it was killed.

From the figures of Roche [1932], it would appear that the mean position of the α -bands of the muscle oxyhaemoglobins of the horse, sheep, ox and dog are very nearly the same, whilst that of the muscle oxyhaemoglobin of the pig is some 4–5 Å. further towards the violet end of the spectrum. Observations on blood oxyhaemoglobin of the pig showed that the α -band here too is some 5 Å. further towards the violet than is that of the oxyhaemoglobin of horse blood. On the basis of these observations, Fig. 1 has been applied directly in calculating the relative concentrations of the haemoglobins in extracts from ox, sheep and dog muscle and has been displaced 5 Å. in calculating them in extracts of pig muscle. The absolute concentrations reported are the mean of the figures obtained at 5600 and 5400 Å. The figures obtained at 5400 Å. were always higher than those at 5600 Å., the difference ranging from 3 to 15%. This is probably due in part to the presence of methaemoglobin and in part to general absorption.

Table I. *Random observations on the concentration of haemoglobin in muscle.*

Tissue	Dry matter	Mean position of α -band in extract	% muscle Hb of total Hb	Conc. of total Hb, g./100 ml.	Conc. of muscle Hb, g./100 ml.
Ox heart	21.61	5800	76.5	0.80	0.62
		5798	70.5	0.77	0.55
Sheep heart	21.18	5789	45	0.66	0.30
		5790	48	0.67	0.32
Sheep heart	18.78	5790	48	0.51	0.25
		5790	48	0.53	0.25
		5797*	67.5*	0.35*	0.24*
Sheep heart	—	5788	42	—	—
Pig heart	21.75	5790.5	63	0.62	0.39
		5789.5	60	0.59	0.35
Pig heart	21.50	5785	46.5	0.74	0.34
		5786.5	51	0.70	0.36
Pig heart	20.45	5794	74	0.45	0.34
		5794.5	75	0.47	0.35
Dog heart	28.55	5788.5	43	0.52	0.23
		5789.5	46	0.51	0.23
Dog leg—gastrocnemius + soleus	25.72	5797.5	69	0.89	0.62
		5797.5	69	0.86	0.59
Shin beef	26.45	5806	94	0.67	0.63
		5806	94	0.71	0.67
Shin beef	—	5805	91	—	—

* Extracted with distilled water.

The difference in the relative concentrations of blood and muscle haemoglobin in heart and skeletal muscle was unexpected. The "shin beef" (Table I) was obtained after the carcass had been dressed. Under similar conditions Shenk *et al.* [1934] have found that 90–100% of the haemoglobin of the "rib-eye" muscle of the ox is muscle haemoglobin.

The high relative concentration of muscle haemoglobin in skeletal muscle is a fact to be borne in mind in the study of the colour of meat. The actual concentration of muscle haemoglobin found by Shenk *et al.* in the "rib-eye" muscle was 0.30–0.45% of the tissue, though the values are probably somewhat low as water was used as extractant. The extensive figures of Whipple [1926] show an apparent normal concentration of 0.3–0.5% muscle haemoglobin in the heart muscle and 0.6–0.8% in the gastrocnemius muscle of the dog. As mentioned in the introduction and further discussed below, it is also somewhat doubtful exactly how close these figures are.

Some observations on Whipple's method of extraction. A sample of tissue was extracted with 0.1% ammonium hydroxide according to the procedure of Whipple [1926]. An absolutely clear filtrate was obtained only after filtering under gravity with a little aluminium hydroxide. At this alkaline reaction there is very little absorption of haemoglobin. 5 ml. of the haemoglobin extract thus obtained were diluted with 5 ml. of water, 2 ml. of approximately *N* HCl added and the solution set aside in the refrigerator. A standard blood acid haematin solution was simultaneously prepared and similarly treated. These acid haematin solutions were colorimetrically compared after 20–24 hours.

Another sample of the tissue was extracted according to the method described above. To 15 ml. of the extract, 2 ml. of approximately *N* HCl were added and,

after 24 hours in the refrigerator, the resulting acid haematin solution was also compared with a simultaneously prepared solution of the blood acid haematin standard. The following are the results:

Concentration in tissue: Whipple's method	...	...	...	13.42x
Concentration in tissue: method of complete extraction	...	...	...	10.55x

where x was the concentration of haemoglobin in the blood solution from which the acid haematin standard was prepared.

Calvo-Criado [1925] has shown that muscle tissue extract causes the breakdown of haemoglobin. Whilst such a breakdown might account for some of this unexpected difference between the results if the velocity of the breakdown were different in the two solutions, almost the whole difference has been traced to another source, that of the unconsidered volume of the tissue in Whipple's method of extraction which becomes considerable owing to the imbibition of fluid. This was shown in the following manner. Approximately 10 g. of sheep heart muscle tissue were made up to 50 ml. with 0.1% ammonium hydroxide and set aside in the refrigerator at 2–4° for 20–24 hours. The mixture was then centrifuged and a known volume of the supernatant fluid pipetted off and filtered clear. The removed haemoglobin solution was replaced by the same volume of 0.1% ammonium hydroxide, the mixture set aside in the refrigerator for a further 20–24 hours, centrifuged and the supernatant fluid filtered clear. The densities of the colour in the two solutions were then compared. From the ratio of the colour densities, it needs but a simple calculation to arrive at the volume of the tissue. The results obtained were

Weight of tissue in g.	Volume in ml.	Volume %
8.4	11.5	23.0
8.4	12.6	25.2
10	12.8	25.6
10	14.1	28.2

* Some of the same sample with which the comparison of the two methods was made.

That imbibition of fluid by the tissue has occurred is apparent, for after centrifuging barely 30 ml. of supernatant fluid were present. It would thus appear that the results given by Whipple's method are somewhat high. It is perhaps of interest that throughout crystal-clear filtrates could only be obtained with the greatest difficulty when Whipple's method of extraction was employed.

SUMMARY.

1. The total haemoglobins of mammalian muscle have been estimated by a spectrophotometric method, and a convenient method of quantitative extraction from well-ground muscle is described.
2. The relative concentrations of blood and muscle haemoglobins in a solution can be determined by the Hartridge reversion spectroscope. The muscle haemoglobin can then be estimated in muscle containing blood.
3. The colorimetric method of estimating haemoglobin by conversion into acid haematin was found suitable for the muscle extract.
4. The method of quantitative extraction gives results about 30% lower than the method of Whipple [1926].
5. A small number of estimations of muscle haemoglobin, chiefly in heart muscle, are reported. Less muscle haemoglobin was found in the bullock's heart than in shin beef.

In acknowledgement, it may be mentioned that Mr J. Hammond, who was interested in the part muscle haemoglobin might play in the colour of meat, suggested this work. Mr R. Hill throughout gave me the benefit of his experience and I am greatly indebted to him for his interest and advice.

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