ACTA CHEMICA SCANDINAVICA 2 (1948) 264—289

A Spectrophotometric Method for the Simultaneous Determination of Myoglobin and Hemoglobin in Extracts of Human Muscle

CHRISTIAN de DUVE*

Biochemical Department, Medical Nobel Institute, Stockholm, Sweden

Except for the detailed study made by Whipple and his collaborators [1-3] on dogs, practically no data are available on the myoglobin (Mgb) content of muscle tissue and on its variations. As far as we have been able to ascertain, there are none on that of human muscle. It is true that, in his review, Millikan [4] alludes to a personal communication by Jonxis referring to the Mgb content of the heart and diaphragm of new born infants and, in an article on myodistrophy, Meldolesi [5] quotes the extensive investigations carried out by his assistant De Orchi. Unfortunately, no trace of these two works has been found in the literature.

Myoglobin may not deserve this lack of attention. Its essential physiological importance with respect to muscular function has been clearly demonstrated by Millikan [4] and the possibility of a myoglobin insufficiency underlying unexplained heart- or skeletal muscle-dysfunctions must not be overlooked. A study of the ratio: blood hemoglobin (Hb)/muscle Mgb may also yield useful information as to the relation existing between these closely similar pigments, especially in view of the increasing attention which has been given in recent years to the pathological variations of other hemoproteins. One can almost safely predict that a careful investigation of the Mgb content of heart and peripheral muscle in various pathological cases must bring to light some sort of Mgb pathology and, if it should not, the finding would in itself be of considerable importance, singling out myoglobin from hemoglobin and other essential iron-porphyrin pigments.

* Present address: Department of Physiological Chemistry, University of Louvain, Belgium.

The present work was initiated at the suggestion of Prof. H. Theorell as a preliminary to the above mentioned investigations. Its aim was to provide an analytical tool which, while requiring a minimum amount of tissue and furnishing sufficiently dependable results, would at the same time be simple enough for large scale routine determinations. As will appear from the details given below, this aim has only been reached so far by compromising between these somewhat conflicting exigencies. However, the method as it has been worked out so far, is not less accurate than most other methods applying to tissue extracts and can also be of utility in the determination of *blood-hemoglobin* in muscle extracts. It is hoped that further investigations, to be carried out by Dr. G. Biörck who has assumed the task of making a detailed survey of the possible pathological variations of the Mgb content, may lead to further improvements of the method described. Several suggestions arising from the experience already gained will be made at the end of this paper.

The main difficulty in the estimation of human myoglobin is that hemoglobin-free samples cannot be obtained, as is the case with animals, and that quantitative separation of both pigments cannot be effected without loss (Theorell and de Duve [6]). Determination of their relative concentrations in a mixture can only be made by taking advantage of the small spectral differences existing between the two pigments. By introducing additional precautions into the classical spectrophotometric technique and carefully determining the constants of the pure components, a satisfactory degree of accuracy has been attained. A further complication, which has been recognized by all the authors who have worked in this field, arises from the fact that extracts which are both truly representative of the Mgb content of the tissue and suitable for spectrophotometric determination, are very difficult to obtain. So far these two qualities have not proved entirely compatible. It will be more convenient to deal first with the spectrophotometric technique; the extraction procedure will be described in a second section and the third section will be devoted to an analysis of the results obtained on a variety of samples of both heart and peripheral muscle.

SPECTROPHOTOMETRIC TECHNIQUE

Principle of the method

Two differential methods have been used for the estimation of Mgb in animals. Shenk, Hall and King [7] measured the absorption at 542, 577, and 582 mμ on untreated muscle extracts, which were assumed to contain mixtures of oxy-hemoglobin and oxy-myoglobin. The spectral differences between these two compounds were used as a basis for the calculation of their respective concentrations. Owing to the ease with which myoglobin is auto-oxidized, it is doubted whether all the myoglobin still was present in

oxy-form in their extracts. Watson's [8] method is also based on the difference between the oxy-compounds, but his extracts are freshly reduced and reoxygenated before the reading, which is done with the Hartridge reversion spectroscope. The position of the maximum of the α-band which varies between 577.4 and 580.8 mμ is used for determining the ratio Hb/Mgb in the mixture. The total concentration of pigment is then measured by the somewhat involved process of reading the absorption of the CO-compounds at 560 and 540 mμ. As the extinction-coefficient of CO-Mgb and CO-Hb are not the same at those wave-lengths, a different value, depending on the distribution determined by the first method, has to be used in each case for this calculation. It is not clear why the CO-compounds were not used as well for the determination of the ratio Hb/Mgb as the difference between the absorption maxima of the CO-compounds is much larger than in the case of the O_2-compounds (10 mμ instead of 3.4). Moreover, Watson uses the coefficient of horse-Hb and Mgb for measurements on other animal species, which is probably incorrect.

The present method is based on the difference in light-absorption of the carbon-monoxide compounds of Mgb and Hb, readings being taken at wave-length values particularly suited to the problem at hand. As an independent control, the total concentration of pigments is measured as reduced pyridine-hemochromogen. It will be clear that much of the accuracy of the measurement depends on the perfect reproducibility of the wave-lengths used. In order to ensure this, it was found necessary to check each wave-length before a reading was taken, using a calibrated glass filter. The constants were determined on solutions of known concentration made up from pure Mgb and Hb. The validity of the method was checked on various mixtures of these solutions and the applicability of Beer's law was checked by widely varying the concentrations of the two compounds, both in pure solutions and in mixtures.

Determination of the constants

Material. — *Human myoglobin* was crystallized according to a slight modification of the method described before [6].

Instead of adding solid $(NH_4)_2SO_4$ to the concentrated extract to separate hemoglobin, the extract was dialyzed first against a 50 % and then against a 75 % saturated solution of $(NH_4)_2SO_4$ brought to pH 8 with ammonia. This procedure had the double advantage of further concentrating the extract and removing the acetate and phosphate present. A known concentration of neutral salt was reached in this way. After centrifuging away the bulk of the Hb, the concentration of $(NH_4)_2SO_4$ was brought to 79 % saturation. The small precipitate formed contained a mixture of Hb and Mgb. Above this point the solution was essentially free of Hb and further treated in the way already described.

After recrystallization, the sample proved homogenous both in the ultra-centrifuge and in the Tiselius apparatus. The iron content was 0.34 %, the nitrogen content 16.8 % of the dry weight.

Human Hemoglobin was prepared from fresh washed erythrocytes, hemolyzed with distilled water saturated with CO_2, the cell-debris being removed by centrifugation. The sample was electrophoretically homogenous and contained 0.34 % iron, and 16.6 % nitrogen.

The spectrophotometric constants for the pyridine-hemochromogens and for the CO-compounds of Hb and Mgb were determined on solutions prepared from these samples and standardized by simultaneous iron, nitrogen, and sometimes dry-weight-determinations.

Apparatus and reproducibility of wave-lengths. — The Beckmann spectrophotometer was used. This apparatus was found extremely reliable as far as the absolute value of the readings was concerned. However, it was observed in the course of this work, that even when the apparatus is perfectly calibrated, the wave-length scale is not entirely reproducible at the higher wave-lengths. In the region between 550 and 600 mμ, shifts of 1 to 2 mμ may occur. These shifts do not affect the entire scale in the same way and are probably due to a very slight unavoidable looseness in the mechanical parts of the monochromator. Whereas no change can be detected in the ultra-violet region, where the dispersion of the prism is greatest, the corresponding discrepancy between the position of the scale and that of the prism will become more and more perceptible, the smaller the dispersion of the prism.

In most cases, a slight error as to the wave-length will not considerably affect the accuracy of a measurement, but, in the present instance, perfect reproducibility of the wave-length was an essential condition. In order to insure this, the following device was used:

A glass light-filter showing a suitable absorption-spectrum (see p. 271) was mounted vertically on a stand fitting into the movable plate normally supporting the cell-holder in the Beckmann apparatus. The extinction-value of the filter was then determined against air at every required wave-length, measurements being carried out simultaneously on the standard solutions without altering the position of the monochromator. The spectra of CO-Mgb, and CO-Hb and of the filter were thus determined, every single set of three points being perfectly matched as to the wave-length. Any desired wave-length corresponding to one of the standard readings could then be obtained independently of the scale itself by first inserting the filter and moving the monochromator in the neighborhood of the required wave-length until the extinction-value previously obtained was reproduced.

The reliability of this simple procedure has been repeatedly checked and it has proved very useful in other spectrophotometric determinations as well. It requires three conditions: 1. The light-absorption of the filter must vary sufficiently with the wave-length in the range where readings have to be made; the steeper the spectral curve of the filter, the more accurate the wave-length adjustment will be. 2. The position of the filter in respect to the light-beam must be kept constant. 3. The spectrum of the filter must remain constant.

The filter used in this work was Seitz VG 20, 1 mm. Its spectrum is given in Fig. 2, along with the standard curves of CO-Hb and CO-Mgb. It is not ideally adapted to the present case as its spectrum shows only a small slope in the region of 568 mμ, where a reading has to be taken. A consideration of the shape of the standard curves will show, however, that a small error in wave-length at that point will not affect the accuracy of the determination in a significant way. On the other hand, at 583.8 mμ, where high accuracy in the wave-length is essential, the slope of the filter's spectrum is such that the wave-length can be reproduced within 1 Å.

The stability of the filter's spectrum can be checked from time to time against a solution of CO-Hb. The concentration of this solution need not be known accurately as the shape of the curve of CO-Hb with respect to the absolute values of the filter will show whether these values have suffered any significant change.

Nomenclature. — In expressing the extinction-coefficient, the classical formula: $\varepsilon = \frac{1}{cd} \log \frac{I_0}{I}$ was used.

d was 1 cm in all cases.
c was expressed in milligrams of dry substance/ml.

The dry equivalent weight of both compounds being 16.200/atom of iron, the equivalent absorption coefficient β can be calculated from the values of ε listed below by the equation: $\beta = 2.3 \times 16.200 \times \varepsilon \times 10^3$ and the equivalent extinction-coefficient by multiplying ε by 16.200.

Furthermore, the values of ε for reduced pyridine-hemochromogen are expressed in mg of dry Hb or Mgb/l ml of the solution used. As this solution is diluted four times for the reading, the true values of molar ε and β of the pyridine-hemochromogen itself will be obtained by multiplying those values, calculated as above, by 4.

Pyridine-hemochromogen standard. — The samples were prepared by adding 3 ml of a solution containing: pyridine 100 ml, *N* NaOH 30 ml, *aq. dist. ad* 300 ml., to 1 ml of the standard Mgb or Hb solution directly in a 1 cm cuvette,

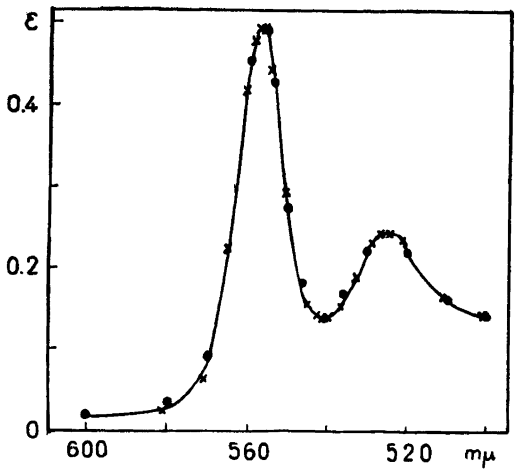

Fig. 1. Spectrum of reduced pyridine-hemochromogen.

● *From hemoglobin*
× *From myoglobin*

and mixing thoroughly with a thin glass rod. Oxidized pyridine-hemochromogen is formed, which is a relatively stable compound. Immediately before taking the spectrophotometric reading, an excess of solid $Na_2S_2O_4$ is added and quickly dissolved by stirring. The reduced hemochromogen is not stable for more than 5 to 10 min and readings have to be taken quickly.

Fig. 1 reproduces graphically the standard values for reduced pyridine-hemochromogen from hemoglobin and from myoglobin between 500 and 600 mμ. It is clear that they are undistinguishable.

As a means of determining protohemin quantitatively, the pyridine-hemochromogen method is not very trustworthy. Even with pure solutions, the error easily attains 2 %. In order to obtain reproducible results, it is essential that the solutions should always be prepared in the same way. We have found the method described above much more reliable than the usual one which consists in splitting off the hemin first with alkali and adding pyridine afterwards. A sufficient excess of hydrosulphite should also be used.

For quantitative measurements readings are usually taken at 557.5 mμ (max.). A reading may eventually also be taken at 540 mμ (min.), the difference between the absorption at both wave-lengths being used in the latter case. The constants for these two wave-lengths are given in Table 1.

Standard extinction values of the carbon-monoxide compounds. — The carbon-monoxide compounds of Hb and Mgb were prepared immediately before the readings, using the apparatus described previously [6] and pure CO. The gas was first allowed to bubble through the solution during 5 minutes, then an

Table 1. Constants of reduced pyridine-hemochromogen. (All values expressed for 1 cm cell-depth.)

Wave-length	Max. 557.5 mμ	Min. 540 mμ	Difference 557.5 mμ—540 mμ
$\varepsilon = \log I_0/I$ for 1 mg Hb or Mgb dissolved in 4 ml pyr-NaOH-hydrosulph.	0.494	0.139*	0.355*
Molar extinction coeffic. $\varepsilon = \log I_0/I$ for $c = 1$ mM red. pyr.-hemochr. p. l.	32.0	9.0	23.0
Molar absorption coeffic. $\beta = \ln I_0/I$ for $c = 1$ mole red. pyr.-hemochr. p. ml. $\times 10^{-7}$	7.36	2.07	5.29

excess of $Na_2S_2O_4$ was added under CO atmosphere and carbon-monoxide led again during 5 more minutes. The solution was quickly transferred into the cuvette, care being taken to fill the cuvette as completely as possible, and to cover it immediately.

At each wave-length, readings were taken simultaneously on CO-Mgb, CO-Hb and on the filter, as described above. The solutions had been brought to identical concentrations in order to enable an accurate determination of the isobestic wave-lengths. The spectra of both compounds and of the filter are given in Fig. 2 and the numerical values of the extinction coefficients have been listed in Table 2 in order to facilitate reproduction of the method. A glass filter of the same type or any other suitable and stable standard can be used for this purpose and it will be sufficient to standardize it against a solution of CO-Hb, as the corresponding values for CO-Mgb can then be obtained by interpolation from the chart below.

In our previous communication on human myoglobin [6] a spectrum of CO-Mgb significantly different from the one published in this paper has been recorded. The values put forward previously rested on a single experiment which, for technical reasons, could not be repeated, and were not given as definitive. Three peculiarities had been observed: 1) The extinction values were uncommonly low compared to those of horse myoglobin. These values had been calculated on the basis of the iron content of the solutions, deter-

* In crude muscle extracts, the absorption at 540 mμ is somewhat raised, probably owing to the impurities present. Better agreement is obtained with $\varepsilon_{(540)} = 0.156$ and $\varepsilon_{(557.5)} - \varepsilon_{(540)} = 0.338$. This empirical value was used for calculation of results on extracts with the aid of formula (7): see further.

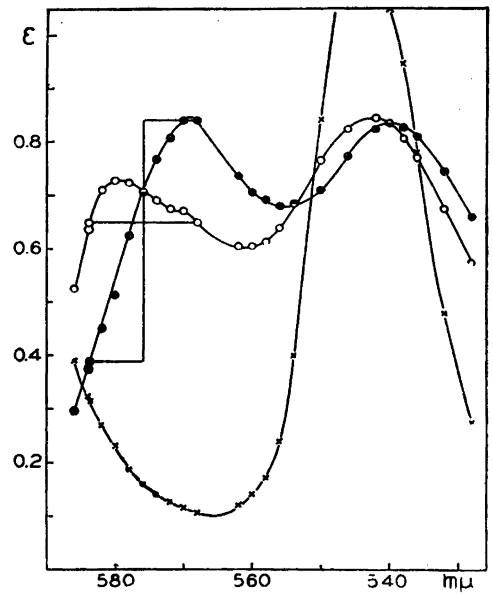

Fig. 2. Spectra of the carbon-monoxide compounds.

●——● *Hemoglobin*
○——○ *Myoglobin*
×——× *Calibration filter*

Table 2. Constants of CO-Hb and CO-Mgb: log I_0/I for d = 1 cm and c = 1 g dry pigment (1/16.200 equivalent)/liter.

mμ	CO-Hb	CO-Mgb	mμ	CO-Hb	CO-Mgb
586	0.296	0.525	560	0.706	0.605
584	0.375	0.636	558	0.693	0.613
583.8	0.388	0.649	556	0.680	0.640
582	0.451	0.710	553.8	0.685	0.685
580	0.513	0.727	550	0.710	0.766
578	0.625	0.723	546	0.773	0.824
575.7	0.706	0.706	542	0.824	0.844
574	0.767	0.690	540	0.833	0.833
572	0.806	0.675	538	0.826	0.806
570	0.839	0.671	536	0.809	0.769
568	0.840	0.649	532	0.744	0.675
562	0.736	0.605	528	0.659	0.574

mined *after the readings*. It has now been ascertained that the added sodium hydrosulphite contained traces of iron, sufficient to decrease the calculated coefficients by about 15 %. The true values approach closely those of horse myoglobin. 2) The peak of the alpha-band was found to be at 577 mμ whereas the corresponding value for myoglobins of other species ranges between 579 and 580 mμ. It will be clear from the dis-

cussion given above that differences of 2 mμ cannot be considered as significant with the type of apparatus used. In the course of a great number of observations on human Mgb, using the Beckmann spectrophotometer, the peak of the alpha-band has been found at every point between 577 and 580 mμ. The mean value of 578.5 mμ has been adopted here. 3) The dissymetry of the two bands has been confirmed. Moreover a faint band has been regularly observed, but with somewhat varying intensity, at 570 mμ. As denatured CO-Mgb has a band at 570 mμ, it is possible that a slight denaturing of the product may be responsible for it.

Stability of the compounds. — CO-Mgb is not as stable as CO-Hb and is relatively rapidly oxidized to met-Mgb in the presence of air. However, if the cuvette is completely filled and quickly covered, this process is sufficiently slow not to interfere with the measurement. After 3 hours, the decrease in extinction at 578 mμ was only 2 %, and the increase at 635 mμ negligible.

Influence of pH. — Between 6.5 and 8, the pH has no measurable influence on the spectral curves. An acid reaction must be avoided, as it tends to accelerate both auto-oxidation and denaturation. In view of the strong acidifying effect of $Na_2S_2O_4$, the solutions must be sufficiently buffered, preferably towards the alkaline range. M/15 Na_2HPO_4 has been used throughout the present work.

Influence of concentration. — Both CO-Hb and CO-Mgb obey Beer's law perfectly within a range of concentration of 0.05 to 1 g/l at the various wave-lengths used for their determination.

Choice of wave-lengths and calculations

Theoretically, the concentrations of Hb and Mgb can be determined by two independent readings taken at two wave-length values where the extinction coefficients of Hb and Mgb are sufficiently different, according to the general formula

$$C_{Mgb} = \frac{A_{(1)}\,\varepsilon_{Hb(2)} - A_{(2)}\,\varepsilon_{Hb(1)}}{\varepsilon_{Mgb(1)}\varepsilon_{Hb(2)} - \varepsilon_{Mgb(2)}\varepsilon_{Hb(1)}} \qquad (1)$$

$$C_{Hb} = \frac{A_{(1)}\,\varepsilon_{Mgb(2)} - A_{(2)}\,\varepsilon_{Mgb(1)}}{\varepsilon_{Hb(1)}\,\varepsilon_{Mgb(2)} - \varepsilon_{Hb(2)}\,\varepsilon_{Mgb(1)}} \qquad (2)$$

where

C_{Mgb} and C_{Hb} are the concentrations of Hb and Mgb
$A_{(1)}$ and $A_{(2)}$ the density values read at wave-length (1) and (2)
ε_{Hb} and ε_{Mgb} the extinction coefficients of Hb and Mgb at both wave-lengths.

The choice of the wave-lengths to be used, which is arbitrary, will be dictated, for obvious reasons, by the shape of the two spectral curves, the points where a variation of the ratio $\frac{C_{Mgb}}{C_{Hb}}$ produces the greatest change in light absorption being the most suitable for the determination of this ratio. In the present case, three points are particularly interesting because, besides satisfying the above requirements, they have the advantage of greatly simplifying the calculations involved and of immediately providing a figure averaging three independent determinations. The first is the isobestic point at 575.7 mμ (see Fig. 2). At this wave-length CO-Mgb and CO-Hb show the same extinction coefficient so that the total concentration in pigment can be determined, irrespective of their relative concentrations, from one single reading at that wave-length, and compared with the value obtained by the reduced pyridine-hemochromogen method. The other two points are 568 and 583.8 mμ (see Fig. 2). At these wave-lengths, CO-Mgb shows identical extinction values and will therefore contribute the same value to the densities read at both wave-lengths. CO-Hb, on the contrary, has widely differing extinction-coefficients and is solely responsible for the difference between the two density values. The concentration of Hb can therefore be calculated directly from this difference irrespective of the amount of Mgb present, and, for that matter, of any other impurity showing approximately the same extinction value at both wave-lengths. The concentration of Mgb will then be obtained as the difference between the value read at the isobestic point and the value obtained for Hb.

In the present case, the following set of equations will be valid

$$C = C_{Mgb} + C_{Hb} = \frac{A_{(575.7)}}{0.706} \tag{3}$$

$$C_{Hb} = \frac{A_{(568)} - A_{(583.8)}}{0.452} \tag{4}$$

$$C_{Mgb} = C - C_{Hb} \tag{5}$$

The value of C can be checked independently by the reduced pyridine-hemochromogen method. If the method described above is applied (1 ml of the solution to be analysed plus 3 ml NaOH-pyridine), a value of C directly comparable with that calculated from equation (3) can be obtained from either of the following expressions

$$C = \frac{A_{(557.5)}}{0.494} \tag{6}$$

$$C = \frac{A_{(557.5)} - A_{(540)}}{0.338} \qquad (7)^*$$

Equation (7) has the advantage of eliminating the error arising from impurities which absorb light equally at 557.5 and at 540 mμ.

Remarks. — It is interesting to note that the values computed from equations (3), (4), and (5) actually average three sets of calculations. With the three readings made, three different combinations of 2 wave-lengths can be used to calculate C_{Hb} and C_{Mgb} according to the general formulae (1) and (2): $A_{(568)} + A_{(575.7)}$; $A_{(583.8)} + A_{(575.7)}$; $A_{(568)} + A_{(583.8)}$. Averaging of the three expressions of C_{Mgb} obtained in this way leads to the following equation

$$C_{Mgb} = 1.3746 \times A_{(575.7)} - 2.1859 \times A_{(568)} + 2.2314 \times A_{(583.8)} \qquad (8)$$

A comparable expression can be obtained from equations (3) and (4)

$$C_{Mgb} = 1.4160 \times A_{(575.7)} - 2.2123 \times A_{(568)} + 2.2123 \times A_{(583.8)} \qquad (9)$$

It is seen that equations (8) and (9) are closely similar and the value of C_{Mgb}, as calculated by the simple expressions (3) and (4) will correspond almost identically to the true mean of the values which could be calculated by combining in the classical way, the values read at 3 different wave-lengths, the only difference being that equation (9) averages the three readings in a slightly different way as compared to equation (8). Both methods of calculation were used on more than 100 sets of determinations on muscle extracts and in no case was the difference between the values obtained more than 1 %. The use of equation (3) and (4) is thereby justified as it leads to greater simplicity without loss of accuracy.

2) Fig. 2 shows the existence of two more isobestic points (553.8 and 540 mμ). Readings at those points might provide a valuable check of the reading at 575.7 mμ, especially as the properties of the filter render these wave-length values very accurately reproducible. Their use is however prohibited in muscle extracts as the disturbing effect of impurities increases at shorter wave-lengths (especially that of cytochrome-c in this particular case). For a similar reason, no attempt was made to take advantage of the differences in the Soret-region.

3) The determination of C_{Hb} is less exposed to error than that of C_{Mgb}, as interference of impurities which like Mgb show the same absorption-value at 568 and at 583.8 mμ is eliminated in equation (3). Such impurities will, on the other hand, appear as Mgb. Unfortunately, the spectral curves do not favor a reverse combination, whereby C_{Mgb} could be calculated directly from the difference in densities at two points at which Hb contributes equally. The most favorable combination of that sort would be 575.7 and 560 mμ, but even there the difference brought about by Mgb is very small and moreover, even small amounts of cytochrome-c will cause a substantial error, as was shown by a few trial tests made on muscle extracts.

---

* For pure solutions $C = \frac{A_{(557.5)} - A_{(540)}}{0.355}$: see previous footnote, p. 270.

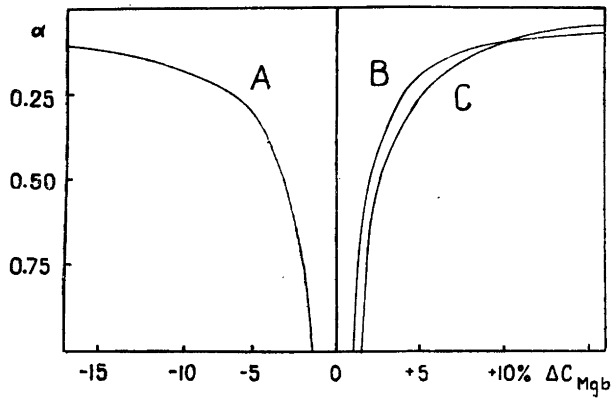

Fig. 3. Percentage of error of C_{Mgb} ($\triangle C_{Mgb}$) *as a function of* $\alpha = \frac{C_{Mgb}}{C}$.

A = If an error of + 1 % is made in $A_{(568)}$
B = » » » » » » » » » $A_{(575.7)}$
C = » » » » » » » » » $A_{(583.8)}$

Evaluation of the error

The present discussion will be limited to a theoretical evaluation of the error inherent to the method itself, applied under the best possible conditions. The close similarity of the two components between which simple spectrophotometry is asked to differentiate, can be expected to assign unavoidable limitations to the accuracy of the determination. Therefore, a range of error larger than the one affecting usual photometric measurements may be predicted. Errors in the extinction value obtained, arising either from personal errors or technical defects of the apparatus, will generally be magnified in the value calculated for C_{Mgb} and it is interesting, in order to define the degree of accuracy of the method, to estimate the extent of this magnification. In this respect, two factors are of importance in the present case: 1) the ratio $\frac{C_{Mgb}}{C}$; 2) the accuracy of the wave-length setting. Furthermore, in order to make it possible to evaluate the applicability of the method to crude muscle extract, where unknown factors may influence the measurements in an uncontrollable way, an independent check of the reliability of each determination will be found necessary. Such a check will be provided by a comparison between the value of C obtained at the isobestic wave-length and that given by the pyridine-hemochromogen method.

Influence of the ratio $\frac{C_{Mgb}}{C}$. — It is obvious that the error affecting the value of C_{Mgb} will be greater, the smaller the ratio $\alpha = \frac{C_{Mgb}}{C}$. Fig. 3 shows the variation of the percentage of error of C_{Mgb}, ΔC_{Mgb} as a function of the relative concentration α of myoglobin in the mixture, in three simple theoretical cases. Curves A, B, and C apply to an error of + 1 % in the estimation of respectively $A_{(568)}$ $A_{(575.7)}$, and $A_{(583.8)}$, the other two values being in each case assumed to be the correct ones. It is seen that the error in the readings are magnified by a factor which is roughly equal to $\pm \frac{1.5}{\alpha}$. If sufficient care is taken, reading errors should not exceed $\pm$ 1 %. Therefore, the value $\pm \frac{1.5}{\alpha}$ % can be taken as the margin of error of the determination of C_{Mbg}, at least if no error is made in the setting of the wave-lengths.

Influence of the wave-length setting. — Assuming the extinction-coefficient of the filter to remain constant and to be measurable within $\pm$ 1 %, the wave-length can be reproduced within 0.2 mμ at 568 mμ, 0.15 mμ at 575.7 mμ, and 0.1 mμ at 583.8 mμ. The maximal error arising from such wave-length shifts will be 0.3 % at 568 mμ, 0.75 % at 575.7 mμ and 1 % at 583.8 mμ, giving a mean maximal error of 0.7 %. It follows that the error in the extinction-values can reach 1.7 %, and the total maximal error in the determination of C_{Mgb} will be $\pm \frac{1.7 \times 1.5}{\alpha} = \pm \frac{2.5}{\alpha}$ %. This value characterizes the range of deviation of a given value of C_{Mgb}.

Test of reliability. — Application of this method to crude muscle extract introduces new possible errors in the evaluation of C_{Mgb}. Impurities may influence the spectrophotometric data either by being themselves light-absorbing in the wave-length region used, or by increasing the natural instability of the hemoglobins and catalysing their transformation into compounds of different spectral characteristics. The extraction procedure itself might also be responsible for similar changes. Unless the resulting modifications of the absorption-values are very important, a plausible value for C_{Mgb} may nevertheless be obtained from the spectrophotometric data. In most cases, however, errors in the data obtained on the CO-compounds will be reflected in a discrepancy between the value of C obtained at the isobestic point and that furnished by the pyridine-hemochromogen determination.* Agreement between these two

* It has been ascertained, for instance, that CO-myoglobin subjected to mild denaturing agents (increasing acidity-concentrated urea) shows a variety of spectra closely resembling those of mixtures of undenatured CO-Mgb and CO-Hb. But, for the same amount of hemin, the extinction-coefficients of the denatured Mgb are significantly lower than those of undenatured mixtures.

values will generally mean that the amount of unmodified protoheme which can be extracted, is present in the solution of CO-compounds as native CO-Mgb and CO-Hb and that interference by impurities is negligible.

Practically, the following test is suggested for the reliability of a given determination. If the pyridine-hemochromogen value agrees within 3 % with the corresponding value obtained at 575.7 mμ on the CO-compounds, it is felt that the whole set of determinations carried out on the CO-compounds can be considered as reliable and the resulting value of C_{Mgb} obtained will be labeled R. A discrepancy of 3—6 % between the pyridine-hemochromogen and the isobestic values should not yet be viewed too seriously, in view of the sensitivity of the test and of the somewhat wide range of error to be admitted in any case. Values of C_{Mgb} falling in this group have been labeled A for acceptable. If the discrepancy is greater than 6 %, the value of C_{Mgb} must be considered as unreliable (U).

A similar classification can also be made, using the difference between the two pyridine-hemochromogen values calculated from equations (6) and (7), on the principle that a significant disagreement between both values tends to cast a doubt on their validity, and, consequently, on the validity of the whole determination.

Judging from the numerous determinations which have been carried out on various mixtures of pure Hb and Mgb, it is felt that this estimation of the error inherent to the method is a generous one. In fact, the practical error in these determinations was usually much smaller than the one allowed theoretically, probably because, in most cases, the errors made tend to cancel each other to a great extent. However, the rule which has been set will be found useful in analyzing the results obtained on extracts where greater discrepancies occur.

EXTRACTION TECHNIQUE

To be suitable for spectrophotometric analysis according to the technique outlined in the previous section, 6 ml of a clear, well buffered, and slightly alkaline (M/15 Na_2HPO_4) solution containing between 0.3 and 0.8 mg of total pigment per ml are required.

Several difficulties were encountered in the attempt to prepare muscle extracts conforming to this rule. When water is used as extracting medium on ground muscle, the extract is invariably turbid and cannot be clarified by centrifuging. None of the reagents which were tried in order to remove this turbidity proved satisfactory. Whenever clarification was achieved, some pigment was carried down and could not be recovered. It was found however

that if a slightly acid medium is used for extraction, clear solutions can be obtained. In order to retain the plasmolytic effect of H_2O, very dilute solutions of varying acidity were tried and $N/100$ acetate buffer pH 4.5 was found to give satisfactory results, the buffering capacity of the tissue being sufficient to bring the pH up to about 6.

Various grinding techniques were tried, with the aim of minimizing the amount of muscle tissue necessary for the determination, enabling an accurate correlation of the results to a dry-weight basis and making the extraction as quantitative as possible. Homogenizing was found more applicable to small quantities of tissue than grinding with sand, but true homogenates could only be obtained by first subjecting the tough muscle structure to a preliminary disruption by freezing and grinding with dry ice. The advantage of this procedure was that the grinding medium was automatically eliminated and that quantitative recovery of the minced tissue was not necessary, as the dry-weight determination could be made on an aliquot of the homogenate.

Practically, the following method was followed:

Approximately 0.5—1 g of muscle tissue was dissected free of fat and connective tissue and washed free of part of the blood by gently squeezing under tap-water. A mortar with some powdered dry ice was prepared and the piece of muscle cut in small strips which were embedded in the dry ice. After allowing a little while for freezing, the mixture was vigorously ground under addition of dry ice when necessary, until a pink homogenous powder was obtained. Before thawing had set in, this powder was transferred to a pyrex-glass homogenizer and further ground in the tube by means of the glass pestle until thawing was completed. 3 ml of $N/100$ acetate buffer pH 4.5 was added and the mixture was homogenized. This part of the procedure was best carried out by hand, under visual control. About 0.6 ml of the homogenate was then pipetted into a tared weighing-vessel with a ground glass stopper (»pig» type) and the remaining quantity into a tared stoppered centrifuge tube of about 6 ml capacity. Both vessels were carefully weighed and the weighing-vessel was reweighed after standing 12 hours at 110°. The ratio: dry weight/wet weight of this aliquot gave the amount of dry matter/g of homogenate and, by multiplying by this factor the weight of homogenate used for the determination, the amount of dry matter analyzed was obtained.

To the aliquot of the homogenate transferred into the centrifuge-tube 3 ml of acetate buffer was added. After thorough mixing with a glass rod, the sample was centrifuged and the clear supernatant decanted into a 10 ml graduated cylinder. The precipitate was washed twice with acetate buffer and the washings added to the first extract. This part of the procedure had to be carried out carefully, as quantitative recovery of the extract and wash-

ings was essential. The total volume of fluid was then read as accurately as possible, after which the extract and washings were mixed in order to equilibrate the concentration. From now on, losses of fluid became of secondary importance, as only the concentrations needed be determined.

The extract was then carefully examined for the presence of any turbidity or solid particles and eventually recentrifuged until a crystal-clear solution was obtained. In this case, a medicine dropper was used to decant the supernatant, of which only 6 ml were necessary for the subsequent determination. To the clear extract a total of 25 mgm solid $Na_2HPO_4 \cdot 12\ H_2O$ p. ml of fluid was added and dissolved by stirring, bringing the pH to about 8 and the phosphate molarity to about $M/15$. This resulted in a volume increase of 1.2 %. It was essential that a perfectly clear extract be obtained *before the addition of phosphate* as solid particles usually disperse in the more alkaline medium, creating unremovable turbidity.

By carefully following the above procedure, extracts suitable for spectrophotometric determination were regularly obtained. 1 ml of the extract was then pipetted into a 1 cm cuvette for pyridine-hemochromogen determination and the remainder used for conversion into the CO-compounds and analysis, according to the technique described in the previous section. The values of C_{Mgb}, C_{Hb}, and C were converted into mg/100 mg of dry weight by the following formula:

$$\% \text{ Mgb} = C_{Mgb} \frac{1.012 \times V \times 100}{d.\ w.}$$

where V is the volume of the extract.
1.012 the correction factor for the addition of phosphate.
$d.\ w.$ the dry weight of the sample analysed.

RESULTS AND DISCUSSION

A total of 113 single determinations have been performed according to the technique which has been described. In a number of cases, determinations were made in duplicate on aliquots of the *same homogenate* as well as on different pieces of the *same muscle sample*. Detailed results will be only presented in as much as they help to evaluate the degree of accuracy and the reliability of the method used. No attempt will be made to correlate the results obtained with clinical data.

Single determinations

Examination of each single set of results separately can only furnish information as to their reliability, according to the reliability-test described in

the first section. On the basis of the deviation between the pyridine-hemochromogen values obtained from equation (6) and (7), 59 out of 90 measurements were found reliable, 18 acceptable and 13 unreliable. From the deviation between the mean pyridine-hemochromogen value and the isobestic value, it was found that 58 out of 113 determinations could be labeled reliable, 28 acceptable and 27 unreliable. The results of the combined tests are summarized in Table 3.

Table 3. Reliability-test.

Deviation between the 2 pyridine-hemochromogen values.	Deviation between pyridine-hemochromogen value and isobestic value.		
	0—3 % Reliable	3—6 % Acceptable	over 6 % Unreliable
0—3 % Reliable	35	15	9
3—6 % Acceptable	9	6	3
over 6 % Unreliable	2	5	6
Not determined	12	2	9

According to this survey, the number of unreliable determinations is abnormally high (30 %), which seems to suggest that the procedure followed is not as harmless when applied to extracts as it is when pure solutions are used. Excluding reading errors, which could hardly account for such a high percentage, significant discrepancies between the 3 values considered will be caused mainly by the presence of light absorbing impurities or of cloudiness or by partial denaturation of the pigments. There is no doubt that interfering impurities may be present in some extracts, but their influence should show equally in duplicate determinations. As this was not always the case, it is obvious that the experimental procedure itself must in some way be responsible for the observed discrepancies.

The most harmful step may well be the bubbling procedure used for saturating the extracts with CO. Watson [8] reports that this technique invariably provoked turbidity in his extracts. This was only exceptionally the case in the present experiments but the possibility of partial denaturation unaccompanied by cloudiness cannot be excluded. In this respect, it must be emphasized that both human hemoglobin and myoglobin are very unstable

in dilute solutions and this instability may well be enhanced in a crude muscle extract.

It is therefore suggested, as a possible improvement, to substitute a more gentle saturation technique to the one described in this paper, for instance the one advocated by Watson [8] which consists in bringing the reduced solution under pure CO atmosphere and equilibrating by simply rotating the tube (without shaking).

Finally, it may be noted that the preceeding argument partly rests on the assumption that the pyridine-hemochromogen determinations are generally more reliable than the measurements at the isobestic point of the CO-compounds. In view of the delicacy of the pyridine-hemochromogen test, which has been commented upon in the first section, a reverse grading might have been hypothecated. However, it will be seen from the figures given in Table 3 that better agreement is obtained between the 2 pyridine-hemochromogen values than between their mean and the isobestic value. Moreover, consideration of the results obtained in duplicate determinations which will be discussed presently, seems to show that the former values are more trustworthy than the latter. The validity of the pyridine-hemochromogen value has been further checked in a number of cases by a reading at 650 mμ, where pyridine-verdohemochromogen shows a strong absorption-band. The absorption was very low at that wave-length, which seems to indicate that no oxidation of the hemin-group had taken place in the extract.

Duplicate determinations

29 homogenates were analysed in duplicate. These determinations show several interesting features and have for that reason, been recorded fully in Table 4.

It will be seen first that better agreement is generally obtained between the pyridine-hemochromogen values than between the isobestic values. Especially when one or both of the determinations of a set are labeled unreliable, the discrepancy is seen to be generally due to erroneous isobestic values, whereas the pyridine-hemochromogen values show satisfactory agreement.

Surprisingly close agreement is obtained between the two myoglobin values of a duplicate set. Only in 4 cases (*) does the observed deviation exceed the allowed deviation calculated by the formula $\frac{2.5}{\alpha}$ and only in one case (**) is the observed deviation more than twice the allowed deviation. In other words the error involved by the complete succession of steps starting with the weighing of an aliquot of a given homogenate is usually smaller than that

Table 4. Results of duplicate determinations. — All values in mg/100 mg of dry tissue.

No.	Reliability	Total pigment (as pyr.-hem.)		Total pigment (as CO-comp.)		Hemoglobin		Myoglobin		
		Mean	Deviation	Mean	Deviation	Mean	Deviation	Mean	Deviation	Allowed deviation
41/42	R R	4.60	±0.015	4.64	±0.09	2.58	±0.06	2.06	±0.03	±0.11
47/48	R R	3.84	±0.05	3.845	±0.165	1.67	±0.09	2.175	±0.075	±0.095
57/58	R R	6.11	±0.11	6.205	±0.045	3.005	±0.005	3.20	±0.04	±0.16
66/67	R R	1.65	±0.045	1.64	±0.06	0.69	±0.04	0.95	±0.02	±0.04
71/72	R R	2.875	±0.005	2.91	±0.08	1.18	±0.11	1.73	±0.03	±0.075
77/78	R R	3.315	±0.09	3.355	±0.065	1.925	±0.005	1.43	±0.06	±0.08
79/80	R R	2.585	±0	2.59	±0.07	1.345	±0.025	1.245	±0.045	±0.06
105/106	R R	3.00	±0.04	2.985	±0.005	0.055	±0.055	2.93	±0.06	±0.075
107/109	R R	3.03	±0.025	3.025	±0.035	0.13	±0.04	2.895	±0.075	±0.075
53/54	R A	3.25	±0.01	3.21	±0.07	1.56	±0.01	1.65	±0.06	±0.085
61/62	R A	4.55	±0.035	4.63	±0.08	2.455	±0.085	2.175	±0.005	±0.105
63/64	R A **	1.975	±0.045	2.05	±0.02	0.97	±0.09	1.08	±0.11	±0.05
73/74	R A*	2.33	±0.005	2.275	±0.035	1.045	±0.045	1.23	±0.08	±0.06
75/76	R A	3.01	±0.095	2.91	±0.03	1.54	±0	1.37	±0.03	±0.07
81/82	R A*	2.83	±0.025	2.795	±0.075	1.305	±0.155	1.49	±0.08	±0.07
97/99	R A	2.98	±0.06	3.045	±0.015	0.36	±0.03	2.685	±0.045	±0.08
98/100	R A	2.98	±0.09	3.015	±0.025	0.655	±0.035	2.36	±0.01	±0.075
110/111	R A	2.81	±0.01	2.915	±0.045	0.03	±0.01	2.885	±0.035	±0.07
25/26	R U	3.95	±0.34	3.66	±0.16	2.16	±0.07	1.50	±0.09	±0.09
49/50	R U	2.685	±0.035	2.535	±0.045	0.995	±0.105	1.54	±0.06	±0.065
51/52	R U	2.03	±0.03	2.10	±0.11	0.71	±0.12	1.39	±0.01	±0.055
101/102	R U	4.60	±0.025	4.365	±0.195	3.005	±0.245	1.36	±0.05	±0.11
103/104	R U	3.58	±0.015	3.51	±0.07	0.26	±0.02	3.25	±0.05	±0.08
45/46	A A	3.02	±0.04	3.14	±0.06	1.11	±0	2.03	±0.06	±0.08
59/60	A A	6.85	±0.045	7.195	±0.105	4.02	±0.03	3.175	±0.075	±0.175
55/56	A U	2.56	±0.01	2.535	±0.095	0.895	±0.125	1.69	±0.02	±0.065
43/44	U U	1.50	±0.02	1.60	±0	0.68	±0.04	0.92	±0.04	±0.04
69/70	U U*	3.725	±0.015	3.365	±0.065	1.725	±0.205	1.64	±0.14	±0.08
112/113	U U	4.39	±0.075	5.05	±0.15	0.335	±0.065	4.715	±0.085	±0.12

allowed for the spectrophotometric determination only. It is clear that the Mgb-content of a given homogenate can be estimated with good accuracy and that the homogenates made according to the technique described are true homogenates.

* Experiments in which the observed deviation is higher than the allowed deviation.

** Experiments in which the observed deviation is more than twice the allowed deviation.

The fact that the agreement is equally good when one or two of the determinations in a set have to be considered unreliable seems to be a very favorable circumstance. Apparently, when the isobestic value turns out to be higher (turbidity?) or lower (denaturation ?) than the pyridine-hemochromogen value, there seems to be a compensation between the errors made so that a correct Mgb-value is obtained. As will be seen in Table 4, the error mainly reflects in such cases on the Hb-value.

Serial determinations on the same muscle sample

In 20 cases, more than one independent analysis, either single or duplicate, was made on the same muscle sample, the number of analyses ranging from 2 to 4. In these cases where the entire procedure was duplicated, less satisfactory agreement was obtained, as is shown by the figures given in Table 5. Only the values for Mgb-content have been given, as the Hb-content is by definition a variable. The number of determinations refers to the number of *independent* determinations made (S = single, D = duplicates on one homogenate). The observed deviation has been calculated as the sum of the deviations of each result from the mean (in absolute value), divided by the number of determinations. The corresponding allowed deviation is the mean of the allowed deviations $\left(\frac{2.5}{a}\right)$ of each determination.

Table 5 shows that a perfect check (within the allowed deviation) was obtained in 8 out of 20 sets of determinations; 7 showed a satisfactory check (up to twice the allowed deviation); 5 displayed significant discrepancies. Considering the numerous factors involved in an analysis of this kind, these discrepancies should not be viewed too seriously. In most cases the agreement is more satisfactory than that generally obtained in tissue-analyses, and only in extreme cases does the range of error actually approach the 30 % limit usually admitted in this type of determination.

However, in view of the good agreement which can be obtained from the homogenizing step onwards, it was felt that better constancy could be expected in serial analyses and considerable time was devoted to a study of the factors which might be responsible for the observed discrepancies. Obviously, they can only be due to one of the three following reasons: 1) Unequal distribution of Mgb in muscle tissue; 2) Failure to extract Mgb completely; 3) Errors in the determination of dry-weight.

Weighing errors could not account for the observed discrepancies: The dry samples were weighed carefully on a sensitive microbalance and duplicate weighings showed an accuracy of 1—2 %. The possibility of unequal distri-

Table 5. Results of serial determinations. — All values in mg/100 mg of dry tissue. S = Single determinations. D = Duplicate determinations on the same homogenate.

No.	Sample	Source	Number of determinations	Reliability	Myoglobin-content in % of dry matter		
					Mean value	Deviation	Allowed deviation
1	Heart-muscle	New-born	2 S	R/R	0.15	±0.05	±0.365
2	»	»	2 S	R/A	0.655	±0.085	±0.245
3	»*	Adult	2 S	R/R	1.37	±0.07	±0.05
4	»*	»	4 D	RR/RR/RA/RA	1.385	±0.076	±0.07
5	»	»	2 D + 1 S	RR/R/RA	1.02	±0.05	±0.05
6	»	»	2 D	RA/RU	1.595	±0.045	±0.075
7	»**	»	3 D + 1 S	RR/RA/UU/U	1.59	±0.18	±0.07
8	»*	»	4 S	R/A/A/U	1.31	±0.115	±0.09
9	»*	»	2 S	R/U	0.905	±0.045	±0.04
10	»*	»	2 S	R/U	1.265	±0.135	±0.085
11	Abdom.muscle	Biopsy	2 S	R/A	3.05	±0.03	±0.18
12	»**	»	3 D	RR/RA/AA	2.85	±0.675	±0.15
13	»**	»	2 S	A/U	2.80	±0.34	±0.155
14	»	Post-mortem	2 D	RR/AA	2.10	±0.075	±0.09
15	»*	»	2 D	RA/RA	2.52	±0.16	±0.08
16	»	»	2 D	RR/RA	2.89	±0.005	±0.07
17	»*	»	2 D	RR/RU	3.09	±0.16	±0.08
18	»**	»	2 S	R/U	1.64	±0.28	±0.05
19	»**	»	2 D	RU/AU	1.54	±0.15	±0.06
20	»	»	2 S	A/U	1.605	±0.005	±0.055

bution of Mgb in muscle tissue cannot be ruled out a priori but appears improbable: Muscle shows an essentially homogenous color and the samples taken for analysis were taken as free of fat and connective tissue as possible. This possibility should therefore only be accepted by excluding the other two with certainty.

There remains the possibility of incomplete extraction. This is by far the most probable explanation for the observed discrepancies. Moreover, direct evidence was obtained in a number of cases that detectable quantities of hemin remained in the tissue after extraction:

Treatment of the residue on a water-bath at 95° with glacial acetic acid saturated with NaCl gave a slightly colored extract, showing the presence of chlorhemin. In a few cases, it was possible to extract the hemin by neutralizing with concentrated NaOH under cooling and treating the moderately warm

super-saturated solution of sodium acetate with a small amount of pyridine. Most of the hemin went over in the pyridine and could be determined spectrophotometrically. This was not always the case and this procedure often brought about partial destruction of the hemin. Treatment of the tissue directly with pyridine was not found satisfactory as the hemochromogen is extremely rapidly oxidized under those conditions, but, by carrying out this procedure in an evacuated Thunberg-tube, a strong pyridine-hemochromogen band could be observed spectroscopically. Yellowish extracts were also obtained by treating the tissue residue with dilute ammonia, but the strong turbidity of the solution rendered spectrophotometric determination impossible.

In 10 cases, somewhat reliable values for unextracted hemin could be obtained and they were found to be quite high, ranging between 5 and 30 % of the extracted material. That such large quantities of hemin should belong to other hemoproteins different from Mgb or Hb appears very improbable, and the observed discrepancies seem to show that at least part of the unextracted hemin is Mgb-hemin.

Muscle-tissue is notoriously resistant to homogenizing devices and incomplete disruption of the cells could therefore easily be responsible for the observed differences. There is however evidence that such was not the case in the present experiments. Parallel experiments made on tissue extracted according to the method described, and on tissue either thoroughly ground with sand or finely divided with a freezing-microtome and subsequently homogenized furnished similar results and hemin could be detected in the residue in each case. Overnight extraction in the cold did not increase the amount of Mgb extracted. Histological examination performed by Dr. Biorck on a number of homogenates, showed the absence of intact cells. It is therefore felt that the technique used allowed complete disruption of the cells.

The possibility that part of the dissolved pigments may be retained by adsorption to the cell structures is suggested by an observation by Dirken [9] who actually describes the adsorption of Hb by washed minced muscle tissue and further states that the adsorbed pigment cannot be washed away with water but can easily be removed by an isotonic salt solution. In the present experiments, washing of the residue with M/10 phosphate buffer pH 6 did not liberate any of the retained pigment. Moreover, there is no correlation between the loss sustained and the amount of tissue extracted, whereas in Dirken's experiments the amount of Hb adsorbed depended solely on the amount of tissue present.

It seems therefore that the unextracted Mgb (and perhaps also Hb) was truly insoluble and the question then arises whether this insolubility is a natural or an artifical phenomenon.

Native Mgb is obviously an intracellular component and it is further known that both mechanical injuries of the muscles (crush-syndrome myoglobinuria) and an unexplained pathological condition (paralytic myoglobinuria) may release myoglobin from the cell-interior, in which case it appears in the blood-stream and is excreted in the urine. Whether these conditions are essentially associated with alterations of the cell-membrane or with the rupture of a linkage between Mgb and the cell-structure is not known. The possible existence of an insoluble structure-bound form of Mgb cannot therefore be ruled out with certainty and it is not impossible that complete release of Mgb from the muscle structure may necessitate disruption not only of the muscle cells but even of the smaller granules which form the homogenate. It may be pointed out in this connection that the heavier particles in the homogenate generally appeared slightly colored, whereas the smaller fragments seemed to be colorless.

On the other hand it is also quite possible that the solubility of Mgb and Hb may be affected by secondary changes, either occuring spontaneously or produced by some step in the extracting procedure.

The fact that the »insoluble» form can be extracted with dilute ammonia is consistant with either explanation, as ammonia causes complete disruption of the granules but will also dissolve denatured proteins. So is the following observation: An ammonia extract of tissue-residue was clarified by ultracentrifuging. Spectroscopical examinations showed bands typical of a mixture of oxy-Mgb and oxy-Hb. After reduction with $Na_2S_2O_4$ and treatment with CO, the spectrophotometric analysis gave a distribution of 50 % Mgb and 50 % Hb, but the isobestic value was much lower than the pyridine-hemochromogen value. A solution of partly denatured Mgb would furnish a similar result. Neutralization or dialysis of the ammonia extract against dist. H_2O caused precipitation of the pigment, which could not be redissolved in a slightly acid or neutral salt solution but dissolved readily in ammonia or an alkaline buffer of pH 9. These facts seem to support the denaturation theory, but it must be remembered that ammonia itself is a strong denaturing agent.

If the solubility of Mgb were affected by spontaneous post-mortal changes, one would expect these alterations to be reflected in a difference between the values obtained on biopsy-samples and those furnished by post-mortem samples. The limited number of determinations made so far does not make a statistical comparison possible but it will be seen from the values listed in Table 6 that there is no indication of a significant difference of the kind surmised. It seems at least probable that Mgb suffers no rapid post-mortal alterations, as long as the muscle remains *in situ*. This is confirmed by the fact that the time elapsing between the time of death and the excision of the sample does not seem to affect the results in a significant way.

Table 6. Myoglobin content of abdominal muscle.

Post-mortem	Biopsy
3.09	3.05
2.89	2.85
2.52	2.80
2.10	2.64
1.64	2.53
1.605	2.16
1.54	2.06
	1.85
	1.36
	1.36

The influence of prolonged storage in the frozen state was also investigated. After 10 days storage, one sample gave a smaller value, one a slightly higher and one a significantly higher value than the one determined previously. A marked increase was also observed in one case after 4 days' storage. Successive determinations performed one or two days running showed variations in both directions. There is however some indication that too long standing at room temperature of a muscle fragment which has been previously kept frozen may cause denaturation *in situ*. This seems to have been the case in experiments no. 7 and 12 of Table 5, in which respectively 3 and 2 of the analyses first performed gave results in perfect agreement and the last one a significantly lower value.

The evidence presented above does not furnish conclusive proof of either of the two hypotheses propounded but seems to agree best with a combination of both and to suggest the following tentative conclusions: Myoglobin can be taken to be intimately linked to the muscle structure and not to be readily separable from this structure. Preliminary freezing seems to facilitate extraction but also to render Mgb more susceptible to subsequent denaturing *in situ*. Even after freezing, complete extraction can only be effected by very careful grinding of the cell particles.

From these characteristics, the optimal conditions for extraction can be deduced.

CONCLUSION

Careful analysis of the results obtained with the method described in this paper applied under a variety of experimental conditions leads to the following conclusions:

The differential determination of human Hb and Mgb in a mixture of both pigments can be made with good accuracy, using the Beckmann spectrophotometer, provided reproducibility of the wave-lengths can be ensured. The device described in this paper has been found satisfactory.

Spectrophotometric errors are larger in extracts than in pure solutions but affect the value for Hb more than that for Mgb, which shows surprising constancy. The greater cause of error is probably the bubbling procedure used for saturating with CO and the use of a gentler technique has been suggested.

Errors due to incomplete extraction seem to be due to a combination of two factors: incomplete release of Mgb from the cell-structure and denaturation *in situ*. Optimal extraction conditions have only been worked out on a compromise basis. Agents like ammonia, which will release Mgb completely cannot be used because they furnish turbid solutions and moreover exert a denaturing effect. The best procedure appears to be freezing followed by thorough grinding with dry ice and homogenizing, using a slightly acid extracting fluid. Under those conditions, a clear extract is obtained but special care has to be taken because Mgb appears to be more labile *in situ* after the tissue has been frozen and subsequently thawed.

In more than half the determinations performed, the variation between two independent analyses was smaller than 10 %.

In view of the fact that the conditions were purposely varied in the experiments described, it is felt that this figure characterizes the range of error of the method applied under optimal conditions.

SUMMARY

1. Accurate spectrophotometric constants have been determined for the carbonmonoxide compounds of human hemoglobin and myoglobin between 600 and 520 mμ, and for the reduced pyridine hemochromogens formed from these substances in $N/10$ NaOH.

2. A differential spectrophotometric method for the determination of human hemoglobin and myoglobin in mixtures of these pigments has been worked out on the basis of the determined constants.

3. The reliability and degree of accuracy of the method have been discussed theoretically and shown to be satisfactory, using a variety of mixtures of both pigments.

4. A method is described for obtaining clear extracts from human heart- and skeletal muscle-tissue, suitable for spectrophotometric analysis according to the method described.

5. Application of the spectrophotometric method to these extracts was found to furnish results reproducible within the limit of error which has to be admitted on theoretical grounds. Extracts have been found, however, to require more careful treatment than pure solutions, and suggestions as to possible improvements have been made accordingly.

6. Serial determinations performed independently on the same samples of muscle tissue gave satisfactory agreement in more than half the cases. Significant discrepancies occured in one fourth of the measurements and were found to be due to incomplete extraction of the pigment which either remained bound to the tissue-granules or had suffered denaturation *in situ*.

7. The factors responsible for incomplete extraction have been investigated and the optimal extracting conditions deduced from this analysis.

8. On the basis of the results obtained so far it is believed that the method, applied under optimal conditions, will allow to determine the Mgb-content of muscle tissue with less than 10 % error.

The author wishes to express his very sincere thanks to Professor Hugo Theorell for his constant help and invaluable advice in the planning and the performance of this work, as well as for the generous hospitality which was extended to him at the Medical Nobel Institute, Stockholm. The kind support of Professor G. Nylin is also gratefully acknowledged.

Special thanks are due to Dr. R. K. Bonnichsen and Ing. Å. Åkeson for their friendly help and particularly to Dr. Gunnar Biörck, who not only provided the necessary specimens but also actively collaborated in the final adjustment of the method described. The technical assistance of Miss L. Rudberg has been invaluable in the performance of a great number of the analyses.

The cost of this work was partly supported by a grant from *Stiftelsen Therese och Johan Andersons minne*.

REFERENCES

1. Whipple, G. H. *Am. J. Physiol.* **76** (1926) 693.
2. Whipple, G. H. *Am. J. Physiol.* **76** (1926) 708.
3. Whipple, G. H., and Robscheit-Robbins, F. S. *Am. J. Physiol.* **78** (1926) 675.
4. Millikan, G. A. *Physiol. Rev.* **19** (1939) 501.
5. Meldolesi, G. *Deut. med. Wochschr.* **63 II** (1937) 1654.
6. Theorell, H., and de Duve, Chr. *Arch. Biochem.* **12** (1947) 113.
7. Shenk, J. H., Hall, J. L., and King, H. H. *J. Biol. Chem.* **105** (1934) 741.
8. Watson, R. H. *Biochem. J.* **29** (1935) 2114.
9. Dirken, M. N. J. *J. Physiol. London* **67** (1929) 282.

Received February 12, 1948.