CL. *POST-MORTEM* FORMATION OF METHAEMOGLOBIN IN RED MUSCLE.

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THE colour of a red muscle is due to haemoglobin present (*a*) as muscle-haemoglobin and (*b*) in any blood corpuscles remaining in the capillaries. The amount of muscle-haemoglobin appears to be independent of the degree of blood removal and dependent on the type of muscle and the age and condition of the animal [Whipple, 1926]. In lean meat, the tissue used in the present paper, it is probable that muscle-haemoglobin is a large fraction of the total pigment [cf. Whipple, 1926, Table IV].

It has long been known that methaemoglobin is slowly formed on exposure of haemoglobin solutions to oxygen. If muscle has any oxidising mechanism which functions in the absence of oxygen no evidence has been obtained that it is responsible for *post-mortem* methaemoglobin formation¹. It is necessary therefore to consider the conditions governing the penetration of oxygen into the tissue.

The presence of oxygen in the tissue.

The tissue used has been the red flexor muscles of the fore leg of the ox. They were removed from the limb 18 to 24 hours after death and unless otherwise stated kept in a room maintained at 0°.

It was found that this tissue had a small oxygen uptake. Therefore, given sufficient time, a "steady state" must be reached where the depth of oxygen penetration is determined by the rate of diffusion of oxygen into the tissue and the oxygen consumption. With large pieces of tissue the surface can be considered as a plane. This case has been treated by Warburg [1923] and Hill [1928, 2] who have shown that the greatest depth, *d* cm., to which oxygen penetrates is given by the equation

$$d = \sqrt{\frac{2c_0D}{A}} \quad \dots\dots(1),$$

where c_0 = oxygen pressure in atmospheres at the surface of the tissue.

D = diffusion coefficient of O_2 through the tissue expressed in cc. O_2 (0°, 760 mm. Hg) diffusing in 1 min. across 1 cm.² when pressure gradient of O_2 is 1 atmosphere per cm.

A = oxygen uptake of the tissue expressed in cc. O_2 (0°, 760 mm.) consumed by 1 cc. of tissue in 1 min.

¹ No detectable amounts of methaemoglobin were formed when the tissue was stored in pure nitrogen.

A slice of the tissue several cm. square and slightly more than 1.5 mm. thick was cut freehand with a razor and laid between two sheets of plate glass. Two glass strips 1.5 mm. thick were placed between the plates, and the tissue was carefully compressed to this thickness so that oxygen could diffuse only across the edges of the slab (Fig. 1, p. 1395). This arrangement allowed spectroscopic examination of the tissue pigments *in situ* at different distances from the air-tissue interface, using a Zeiss microspectroscope with "pointolite" illumination. The oxygen uptake of the tissue can be demonstrated by placing between the plates a slice previously exposed to air for a short time. It will be found, except in a sharply defined outer red zone, that the oxyhaemoglobin is reduced to the purplish haemoglobin because of oxygen removal by the tissue.

A rough value of d can be obtained from the width of the outer zone. It can be safely assumed that where the characteristic purple colour appears the ratio $\frac{\text{oxyhaemoglobin}}{\text{reduced haemoglobin}}$ is less than unity. If the oxygen dissociation curve of muscle-haemoglobin is similar to that of haemoglobin in solution this ratio is unity at 0° when the partial pressure of oxygen is small; let it be taken as 4 mm. Hg [cf. Brown and Hill, 1923]. The partial pressure, c atmospheres, of oxygen in an element dz of tissue, distance z cm. from the surface, is given by the equation (Warburg)

$$c = \frac{Az^2}{2D} - \frac{Azd}{D} + c_0$$

substituting

$$d^2 = \frac{2c_0D}{A}$$

$$c = c_0 \left(\frac{z}{d} - 1 \right)^2 \quad \dots\dots(2),$$

or

$$\beta^{\frac{1}{2}} = 1 - \alpha \quad \dots\dots(3),$$

where β is the fraction (c/c_0) of the surface pressure of oxygen in the element dz and α is the ratio (z/d) of the distance from the surface of this element to the total distance of oxygen penetration. This ratio for the element where the O_2 pressure is 4 mm. is α where

$$\alpha = 1 - \left(\frac{4}{150} \right)^{\frac{1}{2}} = 0.84.$$

Hence when a steady state is reached the width of the red outer strip is at least 84 % of the total distance of oxygen penetration. For the present purpose a measurement of this width (hereafter denoted d) gives a fair approximation to d . A hand lens and a scale graduated in 0.5 mm. were used and 12 readings were taken on each tissue slice; the mean is probably correct to approximately 10 %.

The width of the red strip reaches a definite value in a few hours at 0° and thereafter only increases slowly. The slow increase is not due to a non-attainment of a "steady state," for the same values of d were obtained (after less than 7 hours) with two halves of a tissue slice, one of which was exposed to oxygen and the other to nitrogen for 2 hours before being placed between the

glass plates. In this case the values were approached from two sides. The increase seems to indicate (cf. equation (1)) a decrease in the rate of oxygen consumption slow in comparison with the rate of diffusion of oxygen.

The effect of previous freezing in air on the depth of oxygen penetration at 0° was determined. The slice was cut from the interior of the frozen muscle and immediately thawed by contact with two glass plates at room temperature. The details of one experiment are given in Table I. The control slice was cut from a portion of the same muscle which had been kept at 0°.

Table I.

Temperature 0°. Thawed tissue previously frozen for 18 hours at -10°.

Hours	<i>d</i> cm.	
	Control tissue	Thawed tissue
14	0.24	0.20
40	0.29	0.25
84	0.37	0.37
111	0.39	0.42
135	0.45	0.52

Similar results were usually obtained, *d* increased slowly at an approximately linear rate and the rates for thawed and untreated muscle were similar. In Table II $k_1 = \left(\frac{d \cdot d}{dt}\right)$ is the linear rate of increase in cm./hr. and d_1 is the value of *d* obtained by extrapolating to zero time (time of thawing). The values are the mean of all the results obtained² (using beef from 16 animals).

Table II.

Temperature 0°.

	d_1	k_1
Control tissue	0.21	0.0019
Thawed tissue	0.19	0.0027

Different temperatures of freezing have been used (from -3° to -20° and also in liquid air) and the duration of time in the frozen state has been varied (up to 3 weeks) but no marked effects were observed.

Increase of temperature decreases the value of d_1 ; this agrees with the usual low temperature coefficient of diffusion as compared with that of a process such as oxygen uptake. Mean values of d_1 at three temperatures were 0°, 0.22 cm.; 5°, 0.16 cm.; 15°, 0.09 cm. (increase in *d* was followed only for a short period at the two higher temperatures).

It is of interest to calculate from equation (1) the rate of oxygen uptake at 0° using the mean value of d_1 from Table II. The value obtained,

$$A = 0.8 \times 10^{-4}$$

¹ The appearance in some cases of a brown discoloration in the oxygen region due to methaemoglobin formation is described later.

² There was a small difference in the behaviour of the different flexor muscles. With the more loosely textured, less pigmented muscle in the deeper part of the foreleg, k_1 was larger than the values in Table II.

is necessarily only approximate¹. After 100 hours' standing at 0° A has fallen to 0.2×10^{-4} . Taking the mean value found by Hill [1928, 1] for the respiration of resting frog's muscle at 20°, $A = 7.8 \times 10^{-4}$, and assuming a temperature coefficient of 2.5 per 10°, for living muscle $A = 1.2 \times 10^{-4}$ at 0°. It is possible that the oxygen consumption in the present case may be due partly to the oxidation of unsaturated constituents of injured cells.

Hill [1928, 2] has considered the case of a thick, isolated, living muscle where the lactic acid formed anaerobically diffuses outward and is destroyed in the oxygen region. In the steady state the depth to which oxygen penetrates should diminish rapidly as the thickness of the muscle increases. With the tissue used in the present work the values of d were independent of the area of the tissue slice (which corresponds to the thickness of the muscle). This suggests either that with this tissue there is no interaction between oxygen and the products of anaerobic activity or more probably that there is little or no anaerobic production of lactic acid.

Formation of methaemoglobin in the tissue.

From the above results it can be concluded that methaemoglobin formation in muscle exposed to air should be confined to a thin surface layer and that freezing and thawing of the tissue in air² has no significant influence on the depth of the layer.

Conant [1923, 1924, 1926, 1928] has shown that methaemoglobin and reduced haemoglobin form a reversible oxidation-reduction system of the ferri-ferrous type. Oxyhaemoglobin is therefore not an intermediate step in the formation of methaemoglobin. Conant suggested the following equation for the oxidation by oxygen

Neill [1925] has pointed out that increase of the oxygen pressure decreases the concentration of the other reactant by the formation of oxyhaemoglobin. There should therefore be an optimum oxygen pressure. Neill and Hastings [1925], using sterile haemoglobin solutions, alone and in the presence of various catalysts, found that the rate of reaction was greatest at an oxygen pressure corresponding approximately to that required for 50 % combination of haemoglobin with oxygen, *i.e.* to a small oxygen pressure.

¹ The value of d_1 , 0.20 cm., was divided by the factor 0.84 to obtain a maximum value for the depth of oxygen penetration, *i.e.* a minimum value for the oxygen uptake. The diffusion coefficient of oxygen through muscle at different temperatures has been determined by Krogh [1918-1919]. The calculated value of A depends of course on the applicability of these values to the tissue used.

² It was found that after freezing in saturated NaCl solution the value of d for the thawed tissue was greatly increased. Apart therefore from the effect of NaCl on methaemoglobin formation the increased accessibility of the muscle pigment to oxygen leads to the formation of a much greater quantity of methaemoglobin.

If a similar dependence on the pressure is assumed for the action of oxygen on muscle haemoglobin it would be expected that the rate of methaemoglobin formation in the oxygen region would be greatest in an element at some distance from the surface. This has been found to be the case.

Methaemoglobin in sufficient amounts can be detected spectroscopically in the presence of the other substances whose spectra are observed on examination of muscle (oxy- and reduced haemoglobin and cytochrome [Keilin, 1925]). Tissue slices were cut from the interior of the muscle as the oxygen region of the original muscle if not used immediately may contain a certain amount of methaemoglobin and may have been altered by factors such as drying, etc. It was also necessary to prevent drying of the edges of the slices during storage. These slices enclosed between two glass plates were stored at 0° and examined spectroscopically at intervals. Methaemoglobin was formed only in the oxygen region and more rapidly in the parts further from the air-tissue surface. The difference in rate at different distances from this surface was great enough to form, after a certain time, a yellow-brown zone (containing mainly methaemoglobin) apparently immediately adjoining the region of reduced haemoglobin, while nearer this surface the tissue was still reddish (Fig. 1).

Fig. 1.

I. Region of reduced haemoglobin.

II. Oxygen region containing brown zone due to methaemoglobin formation.

The width of the brown zone increased with time at a somewhat greater rate than the width of the oxygen region d . On the side further from the surface it increased at the same rate as d , since the band always adjoined the reduced haemoglobin. On the side nearer the surface it spread more slowly and after a certain time became indistinct as the amount of methaemoglobin in the layers nearer the surface increased.

The brown discoloration was usually detectable sooner in the thawed tissue than in the control tissue. In some cases the brown layer d_B was sufficiently well defined to be roughly measurable. Table III contains the details of one of the better experiments, d_R is the width of the reddish layer nearer the surface. In other cases these measurements were not possible and the approximate nature of those quoted must be emphasised.

Table III.

Temperature 0°.				
Tissue	Hours	d	d_R	d_B
Thawed	20	0.26	0.21	0.05
	53	0.35	0.22	0.13
	69	0.38	0.19	0.19
	98	0.46	ca. 0.13	ca. 0.33
	141	0.57	Indefinite	Indefinite
Control	20	0.27	0.27	—
	53	0.32	0.32	—
	69	0.37	< 0.37	—
	98	0.41	0.29	0.12

The results can be expressed in terms of the time required for the appearance of a marked colour change. It is assumed, as is the case, that d_B increases initially at approximately the same rate as d and the maximum value of t when $d_B = 0$ is calculated. Table IV contains these values for several experiments where the thawed tissue had been frozen at -10° for approximately 30 hours; No. 3 is the experiment contained in Table III.

Table IV.

Temperature 0°. t (hours).								
No.	1	2	3	4	5	6	7	Mean
Control tissue	40	60	20	50	90	80	100	60
Thawed tissue	30	10	0	30	60	40	50	30

Different temperatures of freezing have been used and the impression was gained that the ratio t thawed/ t control was nearer unity after freezing at higher temperatures (*e.g.* -3°) than at lower temperatures (*e.g.* -20°).

The values in Table IV are little more than qualitative but it seems permissible to conclude that freezing at -10° and thawing the tissue increases the subsequent rate of methaemoglobin formation.

The relation of oxygen pressure to the rate of formation of methaemoglobin.

An attempt has been made to consider in more detail the relation of oxygen pressure to the rate of formation of methaemoglobin in tissue.

The equilibrium between oxygen and haemoglobin has been expressed by equations (4) and (5). It is now known that this treatment is too simple but, as the equations hold over a fair range of oxygen pressures and the question is still undecided, it seems justifiable to use them in an approximate treatment.

$$\frac{[(\text{H}_3\text{X})_n] \cdot p^n}{[(\text{H}_3\text{X} \cdot \text{O}_2)_n]} = K \quad \text{.....(5).}$$

The oxygen concentration and hence the equilibrium constant is expressed in terms of the partial oxygen pressure.

The concentration of reduced haemoglobin at time t

$$= [a - x] \cdot \frac{K}{K + p^n}$$

where $[a - x]$ is the total concentration of haemoglobin (oxy- and reduced) at time t . When the oxidation of reduced haemoglobin is represented by the equation

the rate of methaemoglobin formation dx/dt is

$$\begin{aligned} \frac{dx}{dt} &= k [(\text{H}_3\text{X})_n]^2 \cdot p^n \\ &= k [a - x]^2 \cdot \frac{K}{(K + p^n)^2} \cdot p^n. \end{aligned}$$

At a constant pressure of oxygen

$$\frac{dx}{dt} = k' [a - x]^2.$$

The optimum pressure of oxygen is obtained by differentiating k' with respect to p and equating to zero

$$\frac{dk'}{dp} = \frac{kK^2np^{n-1}}{(K + p^n)^3} \cdot (K - p^n) = 0$$

$$p_{\text{opt}} = K^{1/n}$$

i.e. the optimum oxygen pressure is such that the concentration of reduced haemoglobin equals the concentration of oxyhaemoglobin¹, as found by Neill and Hastings [1925].

As the oxygen dissociation curve of muscle-haemoglobin is unknown the data of Brown and Hill [1923] on human blood have been taken and the distance from the surface of the tissue element in which the optimum pressure exists has been calculated from equation (3)². Table V contains these distances in terms of the fractional distance $\alpha (=z/d)$. Thus at 0° if the depth of the oxygen region is 1 cm. and the optimum oxygen pressure is 4.0 mm. Hg methaemoglobin should be most rapidly formed at a depth of 0.84 cm.

Table V.

Temperature	p opt.	α opt.
0°	4.0 mm.	0.84 cm.
10	6.4	0.79
20	11.4	0.72

The relative times required for the transformation of a given fraction of the total pigment at different depths, assuming the reaction suggested by Conant, have been calculated. This should give at least a qualitative idea of

¹ Lower pressures are obtained if other reactions are assumed for the oxidation to methaemoglobin.

² The influence of the change in the depth of the oxygen region with time is considered below.

the course to be expected in tissue. In a given element the oxygen concentration is constant¹ and the rate of methaemoglobin formation may be written

$$\frac{dx}{dt} = k' [a - x]^2.$$

The time $t_{0.5}$ taken for transformation of half the total haemoglobin (oxy- and reduced) is obtained by integration and substitution of

$$x = 0.5a,$$

$$t_{0.5} = \frac{1}{k'a}.$$

In any element

$$t_{0.5} = \frac{(K + p^n)^2}{akK^2p^n}.$$

Substituting different values of p corresponding to different fractional depths α the relative times at 0° in Table VI were obtained, the half-time in the optimum element being taken as unity. Two values of n have been used, 1.0 and for comparison 2.2 [cf. Brown and Hill, 1923].

Table VI.

α (cm.)	p mm. Hg	Relative times for 50 % transformation	
		$n=1$	$n=2.2$
0.95	0.38	3.2	45
0.90	1.5	1.3	3
0.84	4	1.0	1
0.68	15	1.5	5
0.50	38	2.9	36
0.29	75	5.2	159
0	150	9.9	726

In both cases there is a sufficient decrease in rate as the distance from the optimum element increases to account for the observed differences.

It has so far been assumed that the oxygen pressure in any element remains constant, *i.e.* the depth of the oxygen region is constant. Since d increases linearly the optimum element moves at a constant rate away from the surface. Increase in d increases the oxygen pressure at any point and increase in p in an optimum element decreases the rate of methaemoglobin formation. It can be seen that the smaller the rate of increase of p the slower is the decrease in the rate of methaemoglobin formation.

It can be shown for an optimum element that

$$\frac{dp}{dt} = \frac{\text{constant}}{d}.$$

Thus the partial pressure of oxygen, c atmospheres, at any point depth z in the oxygen region is given by equation (2)

$$c = c_0 \left(\frac{z}{d} - 1 \right)^2 \quad \dots\dots(2).$$

¹ The amount of oxygen consumed in the pigment change is negligible in comparison with the rate of diffusion of oxygen since both the amount of pigment and the rate of change are small.

Substituting $d = d_1 + k_1 t$ and differentiating

$$\begin{aligned}\frac{dc}{dt} &= -\frac{2c_0k_1z(z-d_1-k_1t)}{(d_1+k_1t)^3} \\ &= -\frac{2c_0k_1z(d-z)}{d^3}.\end{aligned}$$

Considering an optimum element $z = \alpha_{\text{opt}}d$

$$\begin{aligned}\frac{dp}{dt} &= 760 \cdot \frac{dc}{dt} = \frac{1520 \cdot c_0 \cdot k_1 \cdot d^2 \cdot \alpha_{\text{opt}} \cdot (1 - \alpha_{\text{opt}})}{d^3} \\ &= \frac{\text{constant}}{d}.\end{aligned}$$

Hence it follows that in succeeding optimum elements the rate of increase of p is smaller since d has increased. The mean rate of methaemoglobin formation is therefore more nearly maintained at the optimum rate as d increases. At a certain value of d the mean rate may be maintained at a high value for a sufficient time to produce rapidly in a given element (and hence in all succeeding elements) the colour change from red to brown. This appears to explain the increase in width of the brown zone on the side further from the tissue surface at the same rate as the increase in width of the oxygen region.

The general correctness of the suggested relation between oxygen pressure, depth of the oxygen region and methaemoglobin formation is supported by the following experiment. A large muscle was frozen at -10° and placed in cylinder nitrogen at 0° . After 40 hours the surface of the muscle was brown. On cutting a transverse section it was found that a thin brown layer (less than 1 mm. thick) containing mainly methaemoglobin covered the surface; beneath this layer the pigment was in the form of reduced haemoglobin. As cylinder nitrogen contains about 1 % of oxygen the depth of the oxygen region should be approximately four times smaller than in air (cf. equation 1) and as the oxygen pressure in the region (ranging from approximately 8 mm. to zero) lies near to the optimum pressure the rapid formation of a thin brown surface layer can be explained. The surface of a similar muscle after freezing and standing in air for 40 hours at 0° was red.

SUMMARY.

1. Some of the factors concerned in the formation of methaemoglobin in pigmented muscles have been considered.

2. As the formation of methaemoglobin in the tissue used was not observed in the absence of oxygen, the depth of penetration of oxygen into the tissue was investigated. After standing a few hours at 0° a "steady state" was established such that the depth of oxygen penetration was approximately 2 mm.; after 100 hours the depth had increased linearly to approximately 4 mm. The increase in depth was attributed to a slow decrease in the oxygen consumption of the tissue. Freezing and thawing the tissue had no significant effect.

3. Methaemoglobin was slowly formed in the oxygen region of tissue exposed to air and most rapidly at some distance from the tissue-air interface.

Freezing and thawing appeared to increase the rate of methaemoglobin formation.

4. The dependence of the rate on the position in the oxygen region was in qualitative agreement with the expected behaviour.

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