14TH EUROPEAN MEETING
OF MEAT RESEARCH WORKERS
BRNO. CZECHOSLOVAKIA AUGUST 26th – 31st 1968

SECTION D 8

C.L. Walters, R.J. Casselden and A.McM. Taylor

British Food Manufacturing Industries Research Association, Leatherhead, Survey, Great Britain

Soret Analysis of Haem Pigments and its Application to the Products of Nitrite Reduction in Muscle

The spectrophotometric analysis in the visible region of mixtures of haem derivatives is difficult where the spectra, as in the case of oxy- and nitrosylmyoglobin, do not display appreciable differences. Selective procedures, such as the formation of a KCN derivative by metmyoglobin and methaemoglobin, are available for some forms of haem pigment but relatively concentrated solutions are required for this and other visible region techniques, with potential difficulties in the clarification of tissue extracts. The extinction coefficients of haem derivatives at their characteristic single peaks in the Soret region are ten or more times those associated with visible peaks, so that clarification is less of a problem and the sensitivity of response is enhanced.

Oxy- and metmyoglobin (or the corresponding haemoglobin derivatives) can be differentiated spectrophotometrically between themselves and from nitrosylmyoglobin by the formation of a CO complex before and after reduction respectively. Nitrosylmyoglobin can be determined by difference after the conversion of all myoglobin present to this form. To estimate any deoxygenated myoglobin in solution, advantage can be taken in Soret spectra determinations of its selective

combination with oxygen.

Symbols

Abbreviations used for reduced myoglobin (haemoglobin), oxymyoglobin (haemoglobin), metmyoglobin (haemoglobin), carbonylmyoglobin (haemoglobin), nitrosylmyoglobin (haemoglobin) and nitrosylmetmyoglobin are Mb (Hb), MbO_2 (HbO_2), MetMb (Met Hb), MbCO (HbCO), NOMb (NOHb) and NOMetMb respectively.

Materials

Purified pig MetMb (E_{Soret}/E 280 mμ = 4,9) was precipitated from aqueous extracts fo washed minced pigs' hearts between 51 - 57 % w/v $(NH_4)_2SO_4$ after treatment as Theorell (1). Pig MetHb was obtained from Koch-Light Laboratories, Colnbrook, Great Britain. The concentrations of the two pigments were based on values at 630 mμ and pH 6.0 of ϵ_{mM} = 3,5 and $E^{1\%}_{1cm}$ = 90.

For cytochrome c, also from Koch-Light Laboratories, a value of 21 was accepted for ϵ_{mM} reduced - oxidised at 550 mμ (2).

Methods

Interconversions of haem pigments

Pig Mb, MbO_2, MbCO and NOMb and the corresponding haemoglobin forms were prepared from MetMb or MetHb in 0,10 M phosphate buffer pH 6,0 by reduction with $Na_2S_2O_4$ followed by treatment with O_2, CO and KNO_2 respectively. On treatment of Mb or Hb with O_2 at this pH, a small amount of haem pigment is re-converted to the met- form (cf. reference 3); the extent of the consequent loss of MbO_2 or HbO_2 has therefore been determined by the method described in the results section. The conversion of Mb or Hb to COMb and NOMb or the corresponding haemoglobin derivatives is not accompanied by the formation of MetMb or MetHb.

Spectrophotometry of haem pigments

Direct and difference (peak to trough) extinction coefficients were determined from spectra of the pigments at concentrations over the range 0-10 μM in 0,10 M phosphate buffer pH 6,0 using an Optica CF4R automatic recording spectrophotometer with 1 cm cells. In this way, linear relationships were established between extinction values and the millimolar concentrations for each combination of pigments, e.g. MbCO minus MbO_2 or MbCO minus MetMb (Fig. 1).

Samples (3,0 - 6,0 gm) of fine minces of pig quadriceps femoris muscles in 0,20 M phosphate buffer pH 6,0 (3,0 - 6,0 ml) containing chloromycetin (10 mg%) were placed in Thunberg tubes 0,5 - 1,0 ml $NaNO_2$ solution, adjusted to give the required final concentration, was maintained frozen in the side arms to avoid splashing whilst anaerobic conditions were attained by repeated evacuation with shaking and filling with O_2 - free N_2. The effectiveness of this procedure was evidenced by the reduction of added cytochrome c and its maintenance in the reduced condition. After incubation, the products were cooled in an ice bath, clarified by centrifugation (25,000 X g for 20 min at 2°) and the supernatants diluted with 0,10 M phosphate buffer pH 6,0.

Results

Reactions of pig myoglobin derivatives

MbO_2, with a peak in the Soret region at 416 mμ, is converted by CO into MbCO, having an extinction maximum at 423 mμ. MetMb is unaffected by CO until reduced with $Na_2S_2O_4$, when MbCO is produced even in the presence of $NaNO_2$ to at least 1,5 mM. The spectrum of MbCO with reference to the same concentration of MbO_2 comprises a peak at 424 mμ and a trough at 411 mμ (Fig. 2), with a millimolar peak to trough extinction coefficient of 100. That of MbCO with reference to MetMb consists similarly of a peak at 424 mμ and a

trough at 408 mμ (Fig. 1) with a millimolar peak to trough extinction coefficient of 220.

NOMb remains unchanged on CO treatment with and without $Na_2S_2O_4$. Mb responds selectively to oxygenation for short periods, the principal product being MbO_2, although approximately 10 % conversion to MetMb occurs at pH values close to neutrality.

Analytical procedure

Total haem in solution for analysis should generally be restricted to 5 μM to ensure a linear response of extinction to concentration. To achieve a suitable concentration, extracts of skeletal muscle should finally be diluted to a volume in ml of 20 times the weight of tissue in g.

An aliquot of the extract is initially bubbled with CO for one min. A spectrum of the product with reference to the untreated extract will comprise a peak at 424 mμ and a trough at 411 mμ if MbO_2 is present in the original (Fig. 2), and its content can be ascertained from the peak to trough extinction increment, based on a millimolar extinction coefficient of 100.

If Mb is also present in solution under aerobic conditions, the difference curve arising from CO treatment of an aliquot with reference to the original mixture will show evidence of a trough at 439 mμ characteristic of the MbCO minus Mb spectrum (Fig. 2) and distinct from the 424 mμ peak of that of MbCO with reference to MbO_2. In such a case, a preliminary spectrum of the untreated mixture with reference to an oxygenated sample will consist of a peak at 435 mμ and a trough at 412 mμ, from which the Mb content may be calculated from a millimolar peak to trough extinction coefficient of 116 for pig Mb minus MbO_2. The analysis for total MbO_2 should then proceed as above upon the oxygenated mixture.

CO treatment of the reference cell eliminates the MbCO - MbO_2 difference spectrum. The subsequent addition of

$Na_2S_2O_4$ to the test cell in the presence of CO results in a further difference spectrum with reference to the mixture treated with CO only if MetMb is available, comprising a peak at 424 mμ and a trough at 408 mμ (Fig.1). The millimolar peak to trough extinction coefficient for pig MbCO minus MetMb is 220, from which the MetMb content ot the original can be calculated. The small amount of MetMb produced during the oxygenation of Mb at pH 6,0 has been determined in this way in order that due allowance could be made in determining the millimolar extinction coefficient of the MbCO minus MbO_2 difference spectrum.

In order to convert all haem pigment in an extract to NoMb, a crystal of $NaNO_2$ + $Na_2S_2O_4$ should be added to an aliquot. The conversion of Mb, MbO_2 and MetMb to NOMb is virtually instantaneous at pH values below 7; above neutrality, several minutes are required for completion and the intermediate formation of Mb can be observed. Total haem is determined spectrophotometrically from the extinction at the Soret peak of NOMb where $\epsilon^{419\ m\mu}_{mM}$ = 122. Interference by turbidity in this use of a direct Soret spectrum can be detected by the extinction at 470 mμ where ϵ_{mM} for NOMb is comparatively low (= 8,8); the extinction rises again sharply below 390 mμ after the addition of $NaNO_2$ and $Na_2S_2O_4$. The NOMb content of the original extract is calculated as total haem - (Mb + MbO_2 + MetMb).

The variation in extinction increments resulting from the repeated analysis of an individual muscle extract can with ease be restricted to a maximum of 0,03 extinction units, equivalent to 0,3 μM MbO_2 or 0,15 μM MetMb at the most. Repeated extraction of minces of fresh pig quadriceps femoris muscles with 0,10 M phosphate buffer pH 6,0 brought into solution in the second and third extracts approximately 30 % and 10 % respectively of the total haem available in the first extract. The haem compositions of the three extracts were generally in agreement at values of about

80 % MbO_2 + HbO_2 and 20 % MetMb + MetHb.

Analysis of pig haemoglobin derivatives

Relatively small differences have been observed between the Soret spectra of corresponding Hb and Mb derivatives, apart from the position of the peak for HbCO at 419 mμ. The peak to trough extinction coefficients for HbCO minus HbO_2 (peak at 419 mμ and trough at 406 mμ) and HbCO minus MetHb (peak at 420 mμ and trough ar 405 mμ) were 56 and 124 respectively for 1 cm light path of a 1% solution. For pig NOHb, $E^{1\%}_{1\ cm}$ = 67 at 419 mμ.

As a result of the deoxygenation of extracts of pig skeletal muscle on bubbling with a stream of nitrogen, a gradual convestion of MbO_2 to Met Mb proceeds. Duplikate analyses of MbO_2 and MetMb (and the corresponding Hb derivatives) in an extract of a pig quadriceps femoris muscle subjected to deoxygenation for 0 - 3 hours are presented in Table I, in comparison with the pigment contents of an aliquot maintained aerobically for 3 hours, which were virtually unchanged. Even less variation existed in general between individual determinations of MbO_2 or MetMb than the small discrepancies between MbO_2 + MetMb and total haem pigment determined as NOMb, from which figure any NOMb present in an original extract would be extimated.

Interference by cytochromes

Cytochrome oxidase present in extracts of skeletal muscle maintained aerobically will be in the ferric condition and, together with the other cytochromes, it will not respond to CO treatment in MbO_2 determinations. The subsequent inclusion of $Na_2S_2O_4$ as in the estimation of MetMb will result in contributions to difference spectra corresponding to COferrocytocyrome a_3 minus ferricytochrome a_3 and of the ferrous forms of the other cytochromes with reference to their ferric states (e.g. ferrocytochrome c - ferricytochrome c, peak 417 mμ, trough 407 mμ, ϵ_{mM} peak to trough = 82). In the presence of nitrite, nitrosylferri-

cytochrome c can be formed (4); its spectrum with reference to ferricytochrome c again comprises a peak at 417 mμ and a trough at 407 mμ, but with a millimolar peak to trouhg extinction coefficient of 104.

Nevertheless, the cytochrome contents of whole pig skeletal muscle are at least one order of magnitude less than that of myoglobin + haemoglobin. Washed skeletal muscle mitochondria as prepared (4) show no evidence of the oxygen carrying pigments at their α - and β - peaks on reduction with $Na_2S_2O_4$. Similarly, Soret difference spectra of reduced mitochondrial suspensions with reference to oxidised preparations in which cytochrome b was maintained in the reduced condition through the use of antimycin-A (5) also did not reveal absorption appertaining to Mb or Hb at 430 mu.

Application of analysis to mixtures of pig MbO_2 and NOMb

MbO_2 and NOMb were prepared from 50 μM pig MetMb in 0,10 M phosphate buffer pH 6,0 with $Na_2S_2O_4$ and O_2 or 0,15 mM $NaNO_2$ respectively, followed by dilution with the same buffer. Such mixtures invariably contained some MetMb arising from the autoxidation of MbO_2 and / or the effect of any excess $NaNO_2$ remaining after $Na_2S_2O_4$ treatment. Nevertheless, the analyses of mixtures of MbO_2 and NOMb agreed reasonably (Table II) with their theoretical compositions; on average, 15 % of MbO_2 and 7 % of NOMb initially present were found on analysis as MetMb.

Variation of haem products of nitrite reduction in skeletal muscle with period of incubation

Fig. 3 illustrates the fate of endogenous haem pigment during anaerobic incubation at pH 6,0 and 37° of the minces of a pig quadriceps femoris muscle with 0,073 - 0,73 mM $NaNO_2$. Rapid oxidation of much endogenous MbO_2 + HbO_2 to MetMb and MetHb was apparent throughout; the initial residue of MbO_2 + HbO_2 decreased with increasing $NaNO_2$ concentration, falling immediately to zero at a level of

0,73 mM and remaining unaltered over 2 hours. At a $NaNO_2$ concentration of 0,073 mM, a gradual increase of MbO_2 + HbO_2 in the aqueous extracts on analysis in air has consistently been observed between 1 - 2 hr, undoubtedly originating from Mb, or Hb formed in the anaerobic incubations. As estimated by difference, some NOMb + NOHb was formed at zero time, which of necessity included a finite period at 0° for the processing of the products for Soret assay. Continued incubation from 0 min resulted in a steady production of NOMb + NOHb at the expense of available MetMb + MetHb, as would be anticipated from the enzymic character ascribed to their formation (6), the percentage of endogenous haem converted to the nitrosyl- forms after 2 hr being virtually independent of nitrite concentration above 0,073 mM. Residual nitrite remained throughout the incubations from all initial levels employed; in the presence of 0,010 mM residual nitrite from its lowest concentration, the MbO_2 + HbO_2 content of the second extraction in air of the muscle mince with 0,10 M phosphate buffer was 6 % less than that of the first, with a concurrent increase of MetMb + MetHb.

Discussion

The composition of the mixture of haem derivatives present in a tissue sample can be ascertained by reflectance spectrophotometry of the surface or by the analysis of the extracted pigments in solution. Reflectance techniques have been successively employed in detecting, for instance, the reduction of MetMb by ground muscle after treatment with $K_3Fe(CN)_6$, where only two haem pigments are involved and the spectra of the reactant and product are very different (7). However, they are unlikely to be applicable to the differentiation of such derivatives as MbO_2 and NOMb with very similar spectra in the visible region or to the determination of the composition of mixtures of more than two haem components.

When analysis is based upon the extracted pigments in solution, it is necessarily assumed that the composition of the extract is representative of the availability of the various forms of haem in the tissue. No selective extraction of one or more haem compounds from muscle minces has been apparent in the absence of nitrite and thus the analyses of successive extractions were very similar on a percentage basis. In the presence of residual nitrite, however, the loss of MbO_2 and increase of MetMb observed in successive extractions of the same incubation products suggest an enhanced susceptibility of ferrous myoglobin after oxygenation to chemical oxidation by low levels of nitrite. Both the met- and nitrosyl- forms of myoglobin and haemoglobin are relatively stable in the absence of strong illumination, and it is unlikely that they would be altered appreciably during the process of extraction. Nitrosylmetmyoglobin is unstable in air and would appear as metmyoglobin on analysis (8).

The observed formation of NOMb with time at the expense of MetMb produced rapidly on oxidation of MbO_2 with nitrite is in keeping with the sequence of enzyme interactions established as a result of the use of skeletal muscle motochondria (6) and based upon the ability of muscle enzymes to reduce NOMetMb in comparison with their inactivity towards MetMb itself.

Acknowledgments

This work forms part of a research project undertaken with funds generously made available by the U.S. Department of Agriculture under U.S. Public Law 480.

References

1. Theorell, H., Biochem. Z. 252, 1 (1932).
2. Massey, V., Biochim. Biophys. Acta 34, 255 (1959).
3. Yamazaki, I., Yokota, K., and Shikama, K., J. Biol. Chem. 239, 4151 (1964).
4. Walters, C.L., and Taylor, A.McM., Biochim. Biophys. Acta 96, 522 (1965).
5. Thorn, M.B., Biochem. J. 63, 420 (1956).
6. Walters, C.L., Casselden, R.J., and Taylor, A.McM., Biochim. Biophys. Acta 143, 310 (1967).
7. Stewart, M.R., Hutchins, B.K., Zipser, M.W., and Watts, B.M., J. Food Sci. 30, 487 (1965).
8. Fox, J.B., and Thomson, J.S., Biochemistry 2, 465 (1963).

Table I

Analysis in duplicate of MbO_2 and MetMb contents of a pig muscle extract deoxygenated by a stream of nitrogen

1 : 1 w/v aqueous extract of minced quadriceps femoris muscle diluted 1 : 10 v/v with 0,10 M phosphate buffer pH 6,0 and bubbled with nitrogen for periods up to 3 hr at room temperature, with a further aliquot of the diluted extract maintained aerobically for 3 hr.

Time of deoxytenation hr	Concentration of haem pigment µM			Total haem found µM
	MbO_2	MetMb	Total	
0	3,4	0,61	4,0	
	3,3	0,63	3,9	4,1
1	2,4	1,6	4,0	
	2,4	1,6	4,0	4,2
2	1,4	2,7	4,1	
	1,4	2,7	4,1	4,2
3	0,75	3,4	4,2	
	0,80	3,5	4,3	4,2
Stored 3 hr	3,3	0,64	3,9	4,2

Table II

Analysis by soret spectra of the composition of prepared mixtures of pig MbO_2 and NOMb.

+ Percentage composition based on total haem determined.

Component of mixture	Mixture A				Mixture B				Mixture C			
	Nominal composition		Composition determined		Nominal composition		Composition determined		Nominal composition		Composition determined	
	µM	%	µM	%	µM	+%	µM	%	µM	+%	µM	+%
Mb O_2	2,9	25	2,4	21	5,8	50	4,6	42	8,6	75	7,5	67
MetMb	0,0	0	1,2	11	0,0	0	1,0	9	0,0	0	0,9	8
NOMb	8,6	75	7,7	68	5,8	50	5,5	49	2,9	25	2,7	25
Total haem	11,5		11,3		11,6		11,1		11,5		11,1	

Legends to Figures:-

Fig. 1. Soret spectra of 3,8 µM pig met- (MetMb) and carbon monoxide(MbCO)myoglobin at pH 6,0 and of the difference spectrum of 3,8 uM MetMb with reference to 3,8 µM MbCO illustrating the peak to trough extinction difference used to determine millimolar coefficients.

Fig. 2. Difference Soret spectra of 3,6 µM carbon monoxide myoglobin with reference to 3,6 uM oxymyoglobin and to 3,6 µM deoxygenated myoglobin at pH 6,0.

Fig. 3. Percent endogenous haem occurring in oxy- (……), met- (X -----X) and nitrosyl- (_____) forms on anaerobic incubation at pH 6,0 and 37° of mince of pig quadriceps femoris muscle with (A) 0,073 mM, (B) 0,15 mM, (C) 0,44 mM and (D) 0,73 mM sodium nitrite for 0 - 2 hours.

Fig. 1

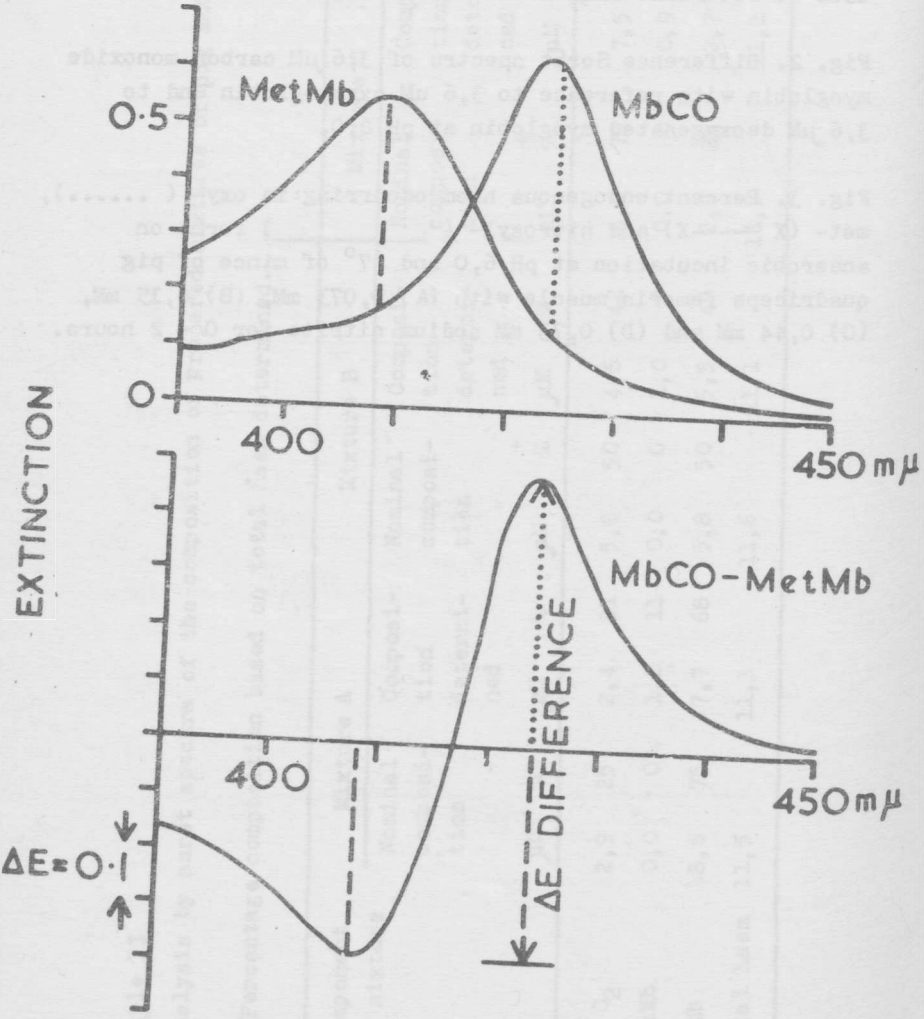
MetMb
MbCO
0·5
0
400
450mμ
EXTINCTION
MbCO-MetMb
ΔE DIFFERENCE
400
450mμ
ΔE=0·1

FIG 2

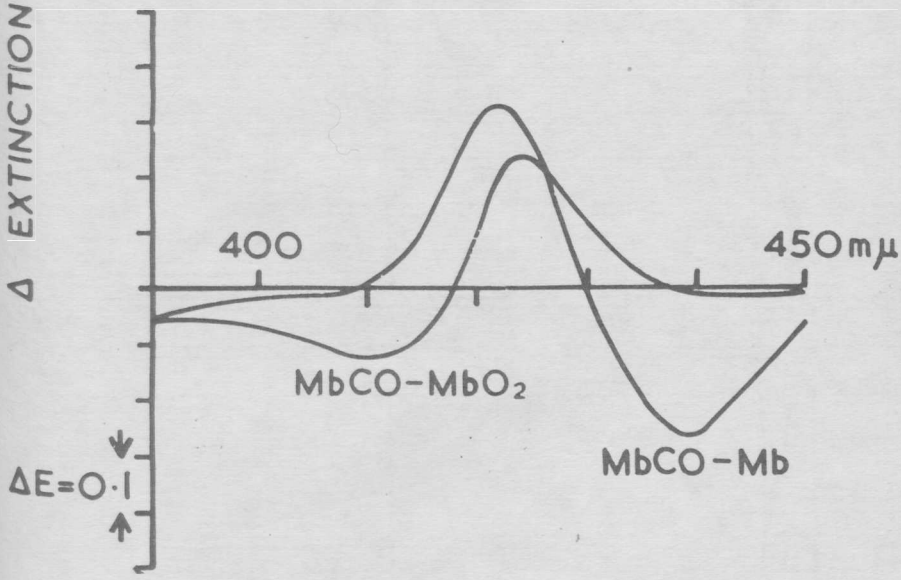

Fig 3