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Mechanism of Acetone Extraction of Haem from Myoglobin in Phosphate Buffer

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ABSTRACT

Phosphate buffer appears to exert a considerable destabilising effect on myoglobin, causing the liberation of haem when the protein is precipitated with acetone. This effect is similar to one reported previously involving haemoglobin. Evidence is presented to suggest that dissociation of the haemoprotein under acetone precipitation conditions is caused by a change in the pH of the medium when acetone is mixed with phosphate buffer.

INTRODUCTION

In our previous paper (Wedzicha & Ladikos, 1985) we reported that, unlike acetate or citrate buffers, phosphate buffer appears to exert a considerable destabilising effect on haemoglobin when the haemoprotein is precipitated with acetone (final composition: acetone, 80%; water, 20%), causing the liberation of haem. The result has important implications for the determination, by acetone precipitation and conversion of liberated haem to acid haematin, of nitrosylhaemoglobin in the presence of haemoglobin in meat and model systems containing phosphate ion. The principal colouring agent in meat is myoglobin and the method of acetone precipitation has been used for the determination of nitrosylmyoglobin. It is, therefore, important to extend the

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investigation of the haem-labilising property of phosphate buffers to their effects on the precipitation of myoglobin. Our studies on this haemoprotein are reported here.

During the course of these investigations it was noted that the visible spectrum of an acetone extract of myoglobin in phosphate buffer was different from that of haemin chloride or haematin in the same solvent. The observation that, by carefully acidifying solutions of haemin chloride or haematin their spectra could be made to resemble the spectrum of the acetone extract, prompted us to consider whether the addition of acetone to phosphate buffers could result in a lowering of the pH of the medium. The dissociation of haemoglobin and myoglobin is promoted by acid conditions (Fronticelli & Bucci, 1963). The second part of this paper describes our attempt to link the apparent lability of the haem in myoglobin in phosphate buffer under acetone precipitation conditions with a change in the pH of the medium.

EXPERIMENTAL

Purity of myoglobin

The myoglobin was sperm whale skeletal muscle myoglobin (Sigma Chemicals Ltd) described as essentially salt free, 95–100%, crystallised and lyophilised. The homogeneity of the sample was examined by disc electrophoresis on polyacrylamide gels (10% with SDS and 5% without SDS). After electrophoresis, the gels were scanned by means of a densitometer at 410 nm, corresponding to the wavelength of the soret peak of the haem moiety. The gels were then stained with Coomassie Blue and, after destaining, scanned at 640 nm. The composition of myoglobin was further examined by determination of (a) the iron content, according to the method of Cameron (1965), (b) the haem content by extraction with acidified acetone as reported previously (Wedzicha & Ladikos, 1985) and (c) the ratio of absorbances at 410 and 280 nm of a dilute solution of myoglobin (Hanania *et al.*, 1966; Hardman *et al.*, 1966).

Acetone precipitation of myoglobin

The procedure adopted for the preparation of solutions of myoglobin, acetone precipitation of myoglobin from buffered media (acetate, citrate

and phosphate buffers) and the subsequent determination of liberated haem as acid haematin, was identical to that described for haemoglobin in our previous paper (Wedzicha & Ladikos, 1985). The results were also corrected for errors due to loss of haem during filtration, as described previously. In the original investigation the final concentration of acetone after precipitation of the protein was a constant 80% and this amount was also present in the solution used for spectrophotometric measurement of acid haematin at 540 nm. In experiments where it was desired to vary the concentration of acetone, the appropriate amount of acetone was added, with gentle mixing, to a convenient volume (2–10 ml) of myoglobin solution. The mixture was centrifuged (1500 × *g*) and an aliquot of the supernatant was acidified with hydrochloric acid (0·2 ml) before making up with acetone and water to give a final composition of 80% acetone, 20% water. The absorbance of the resulting solution was measured at 540 nm. Thus, calibration data for the determination of haem as acid haematin in 80% acetone were also applicable to these experiments.

Ethanol precipitation of myoglobin

It was desired to compare the effects of using acetone and ethanol for precipitation of myoglobin. The procedure involving ethanol was identical to that described for acetone, with the exception that the aqueous ethanolic extract was evaporated to dryness under reduced pressure and redissolved in acidified aqueous acetone (80% acetone) for spectrophotometric measurement. One set of calibration data therefore served experiments involving the use of acetone and ethanol.

Change in pH on adding acetone to phosphate buffer and buffered myoglobin

It was desired to obtain evidence that, when acetone is added to a solution of phosphate buffer, there is a change in the pH of the medium. Unfortunately, the response of the glass electrode in the presence of a large amount of acetone is difficult to define, as are the activities of the various ionic species present in the medium. Direct measurement of pH was therefore not feasible. It was decided to attempt to find evidence of change in pH by the following semiquantitative method. The appropriate amount of acetone was added to a convenient volume (2–10 ml) of phosphate buffer or buffered myoglobin solution with gentle mixing. The

mixture was centrifuged ($1500 \times g$), the supernatant removed and evaporated to dryness under reduced pressure. The resulting solid was dissolved in the same volume of water as the volume of buffer or myoglobin solution used. The pH of this solution was measured using a glass electrode. One such solution was subjected to pH titration with sodium hydroxide (0·02 M).

RESULTS

Purity of myoglobin

Analysis of myoglobin by polyacrylamide gel electrophoresis revealed that the sample was apparently not homogeneous. Scanning of gels (without SDS) at 410 nm showed the presence of at least four haem-containing fractions, with some 70% of the total optical density being found in a main peak. On the other hand, the use of SDS in the gels reduced the electrophoretogram to a single peak with only traces of other components. When stained with Coomassie Blue these other components were stained more readily than the protein comprising the main peak, on the basis of the relative absorbances of the unstained components at 410 nm and the stained components at 640 nm. The apparent heterogeneity of sperm whale myoglobin has been reported in the literature. Hardman *et al.* (1966) found that highly purified sperm whale myoglobin contains five fractions identifiable as myoglobin and our electrophoretograms show patterns similar to those reported by these workers.

A good indicator of freedom from contamination by non-haem proteins is the iron content of the myoglobin. Analysis gave $0{\cdot}300 \pm 0{\cdot}024$ g Fe/100 g myoglobin (mean of ten determinations $\pm$ standard deviation), a result in good agreement with the theoretical value of 0·314 g Fe/100 g myoglobin calculated using a molecular mass of 17 816 daltons (Edmundson & Hirs, 1961). The addition of acetone to a solution of myoglobin in water to give a final acetone content of 80% caused $2{\cdot}5 \pm 0{\cdot}6$% of the available haem to appear in the acetone extract (total available haem = 27 mg per litre as acid haematin in the extract). The protein was considered not to be significantly contaminated by free haem. When determined by extraction with acidified aqueous acetone (80% acetone) the total haem content of the myoglobin was within 5% of the expected value. A sensitive indicator of the quality of myoglobin is the

ratio of absorbance at 410 nm to absorbance at 280 nm. Since the ratio is dependent on pH and ionic strength, it is important that measurements are carried out under the conditions used to obtain literature values. When measured under the conditions used by Hanania *et al.* (1966), our determinations gave a ratio of 5·09 ± 0·06 (mean of eight determinations ± standard deviation) compared with the literature value of 5·03. Hardman *et al.* (1966) quote a value of 5·25 ± 0·05 under the stated condition of pH < 7.

It was concluded that the myoglobin sample was of sufficiently high quality for the studies reported in this paper.

Acetone precipitation of myoglobin

Suitable pH values for model studies of reactions of haemoproteins in meat systems lie at the high end of the acetic acid/acetate ion buffer range, i.e. > pH 5·7, and at the low end of the dihydrogen orthophosphate/monohydrogen orthophosphate ion buffer range, pH 6·0. These pH values may also be covered using citrate buffer and, for this reason, our previous paper (Wedzicha & Ladikos, 1985) was concerned with a comparison of the effects of these three buffer systems on the release of haem during the precipitation, with acetone, of haemoglobin. In this paper the results are extended to myoglobin. In order to provide a direct comparison between the effects of the various electrolytes, the pH of all three buffer systems was set to the same value in the range pH 5·7–5·8 although, at these values, neither the acetate nor the phosphate buffers have much buffering capacity. The pH values reported are measured values in the haemoprotein solution. The ability of phosphate ion to promote the dissociation of myoglobin is clearly demonstrated in Table 1 where each value is the mean of six separate determinations and the errors are standard deviations. The concentrations shown in this Table and elsewhere refer to those of haematin in the acetone extract of myoglobin. Since, in all cases, the myoglobin undergoes a fivefold dilution on the addition of acetone, the equivalent amount of haematin, in the form of myoglobin in the original solution, is five times the value shown, neglecting changes in volume when acetone and water are mixed. In addition to showing the apparently specific haem-labilising property of phosphate buffer, Table 1 illustrates the very significant effect of phosphate buffer concentration and of pH. A high yield of haem is favoured by high buffer concentration and low pH. Examination of Table

TABLE 1

Effect of Components of Acetate, Citrate and Phosphate Buffers and of pH on Release of Haem from Myoglobin in 80 % Acetone (Aqueous). Concentrations Determined as Acid Haematin in Acetone Extract

Buffer	*pH*	*Buffer concentration* (M)	*Per cent extraction*	*Total haematin* (*mg litre*$^{-1}$)
Acetate	5·77	0·10	1·8 ± 0·5	22·1 ± 0·2
Citrate	5·73	0·09	3·4 ± 0·6	29·6 ± 0·3
Phosphate	5·73	0·05	53·0 ± 2·3	18·1 ± 0·1
	5·72	0·10	85·6 ± 2·0	18·0 ± 0·1
	5·71	0·30	92·6 ± 1·2	18·0 ± 0·1
	5·74	0·10	59·2 ± 1·6	34·9 ± 0·1
	6·01	0·10	39·3 ± 1·3	35·1 ± 0·1
	6·31	0·10	13·5 ± 0·7	34·9 ± 0·2

1 also reveals that the yield of haem on extraction depends on the total concentration of myoglobin (compare data for 0·1 M phosphate buffer at total haematin concentrations of 18·0 and 34·9 mg per litre, pH 5·72 and pH 5·74, respectively). This dependence is illustrated in Fig. 1 where, for comparison, the data are superimposed on the data reported in our previous paper for haemoglobin. In both cases the extent of extraction is linearly dependent on the concentration of haemoprotein. The consistently higher extraction of haem from myoglobin than from haemoglobin, despite the fact that the measurements in the case of myoglobin were made at a higher pH (pH 5·72 compared with pH 5·56 in the case of haemoglobin) indicates that myoglobin is significantly more labile under these conditions than is haemoglobin.

Change in pH on adding acetone to phosphate buffer and buffered myoglobin

The possibility of there being a change in the pH of the medium when acetone is added to phosphate buffer is suggested by the following result. If acetone is added to phosphate buffer (pH 5·65, 0·1 M) until the concentration of acetone is 80 % and the solid recovered after evaporation of the supernatant is redissolved in water, the pH of the resulting solution is 2·84. The pH of a solution with a similar amount of sodium dihydrogen

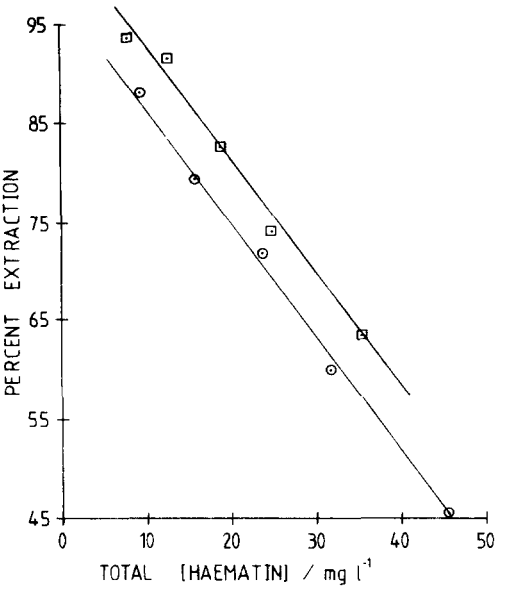

Fig. 1. Effect of concentration of haemoglobin and myoglobin on yield of haem on precipitation with acetone (80% aqueous). Buffer: 0·10M phosphate, pH 5·56 for haemoglobin ⊙, and pH 5·72 for myoglobin ⊡. Concentrations determined as acid haematin in acetone extract.

orthophosphate is in the region of 4·3. The phosphate buffer consisted of a mixture of dihydrogen and monohydrogen orthophosphate ions and a pH value lower than 4·3 after precipitation of some of the phosphate species seems inconceivable. A similar result is obtained when a solution of sodium dihydrogen orthophosphate (0·1M) alone is mixed with acetone and the supernatant treated in the same way. The reason for the lowering of pH can be seen when the recovered solid is subjected to pH titration with sodium hydroxide. Two end-points are evident. The first is due to neutralisation of orthophosphoric acid while the second is due to the conversion of dihydrogen orthophosphate to monohydrogen orthophosphate ion. In the case of the supernatant remaining after precipitation of the phosphate buffer some 17% of the total phosphate was found to be in the form of orthophosphoric acid.

The question which now needs to be answered is whether there is any connection between this change in pH and the liberation of haem from myoglobin. The approach adopted was prompted by the observation that the yield of haem from myoglobin in phosphate buffer was a function of

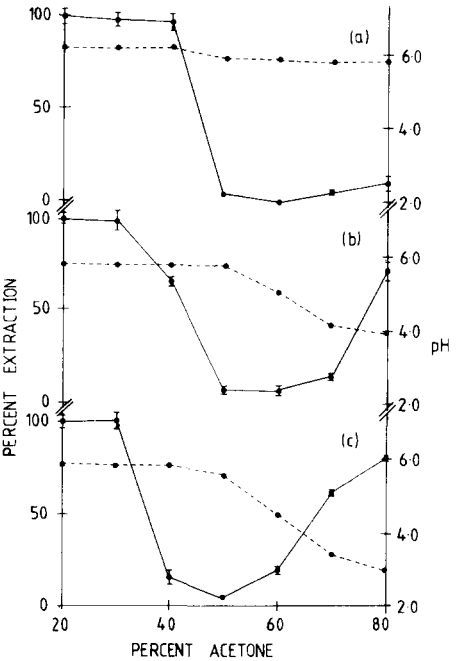

Fig. 2. Effect of acetone concentration and buffer concentration on yield of haem (———) on precipitation of myoglobin from phosphate buffer, and on the pH (- - - - -) of a solution of the solid recovered by evaporation of the aqueous acetone extract. Experimental conditions: (a) 0·005M phosphate buffer, pH 6·15, total [haematin] = 20·1 ± 0·2 mg litre^{-1}; (b) 0·10M phosphate buffer, pH 5·72, total [haematin] = 20·5 ± 0·4 mg litre^{-1}; (c) 0·50M phosphate buffer, pH 5·80, total [haematin] = 20·7 ± 0·1 mg litre^{-1}.

the amount of acetone added. Figure 2 ((a), (b) and (c)) shows the result of increase in the amount of acetone on the yield of haem and on the pH of a solution of the solid recovered from the aqueous acetone supernatant after precipitation of the protein. The experiments were carried out at various buffer concentrations. At low concentrations of acetone (< 30 %) myoglobin is soluble in the mixture and, therefore, all the haem is measured as extractable. As the concentration of acetone is increased above 40 %, the protein is effectively precipitated. In the case of the most concentrated phosphate buffer (0·5M) it is evident that production of orthophosphoric acid in the supernatant takes place above 40 % acetone and gradually increases with increasing acetone content. In the case of 0·1M phosphate buffer the production of acid with increase in acetone content is delayed, as is liberation of haem from myoglobin, when

compared with the effects of the 0·5M buffer. In the case of the most dilute buffer (0·005M) the production of acid is small, if significant, and the amount of haem released is likewise very small.

If this final pH is the variable which determines the amount of haem which is extracted then, for a given final pH, the amount of haem extracted should be independent of the concentration of the buffer used. The data plotted in Figs 2(b, c) allow an approximate estimate of the yield of haem to be made at any pH value shown and some of these results are given in Table 2. It is clear that the yield of haem is consistently higher when precipitation takes place from 0·5M buffer than from 0·1M buffer. The correspondence between the sets of results at the two concentrations is, however, very much better than that obtained when the data for yield are examined with acetone content as the independent variable (compare the graphs shown in Fig. 2). The pH of the medium appears to be a much more important variable than acetone content.

The importance of the pH change was reinforced by two further observations. First, it was found that haem was released from myoglobin when the protein was precipitated from phosphate buffer with ethanol. It was decided, therefore, to compare the effects of acetone and ethanol on the yield of haem on precipitating myoglobin from phosphate buffer (0·1M, pH 5·74) with varying amounts of the two solvents. The range of acetone and ethanol contents in the extracts was, respectively, 70–82% and 78–90%. Figure 3 shows the yield of haem as a function of the pH of a solution of the solid recovered after evaporation of the extract. The error bars represent standard deviations calculated from three measurements. It is evident that, over a major part of the pH range investigated, the data fall on a single curve, irrespective of whether the solvent was acetone or ethanol. Secondly, it was speculated that the pH change which takes place

TABLE 2

Yield of Haem on Precipitation of Myoglobin with Acetone as a Function of the pH of a Solution of the Solid Recovered After Evaporation of the Aqueous Acetone Supernatant. Data Obtained from Graphs shown in Figs 2(b) and (c)

Buffer concentration (M)	*Per cent extraction at pH value shown*			
	3·0	*3·5*	*4·0*	*4·5*
0·10	69	45	21	12
0·50	78	58	37	18

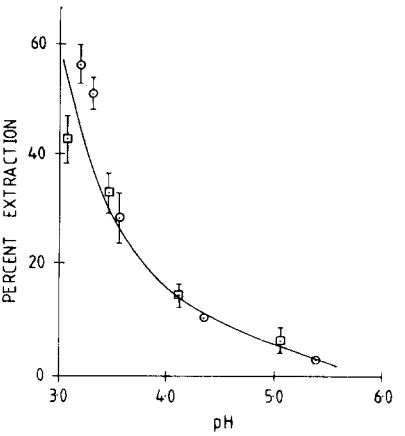

Fig. 3. Yield of haem on precipitation of myoglobin with acetone and ethanol as a function of the pH of a solution of the solid recovered after evaporation of the supernatant. Experimental conditions: 0·10M phosphate buffer, pH 5·74, total [haematin] = 19·4 ± 0·0 mg litre^{-1}. Acetone ⊙. Ethanol ⊡.

on precipitation of phosphate buffer could be prevented by the addition of a buffering component which would not precipitate under the conditions used here. A buffer whose components remain in solution is 0·1M acetate buffer. In order to test whether the addition of acetate buffer would prevent the haem-labilising property of phosphate buffer, the precipitation of myoglobin was carried out from a mixture of equimolar amounts (0·1 M) of acetate and phosphate buffers at pH 5·84. The yield of haem was too small to be measured (< 0·25%).

Since the yield of haem is dependent on the concentration of myoglobin it is of interest to examine whether there is any relationship between the

TABLE 3

Effect of Concentration of Myoglobin on the pH of a Solution of the Solid Recovered after Evaporation of Aqueous Acetone Extract of Myoglobin in Phosphate Buffer. Experimental Conditions: 0·10M Phosphate Buffer, Initial pH, 5·70–5·72.

Total haematin (mg litre^{-1})	*pH*
29·1	2·97 ± 0·01
21·4	2·96 ± 0·00
14·5	2·92 ± 0·00
8·8	2·91 ± 0·01

final pH after precipitation and the concentration of myoglobin used. Data showing the pH of solutions prepared from solids recovered from aqueous acetone extracts of myoglobin in phosphate buffer at constant buffer composition and precipitation conditions, but variable amounts of myoglobin, are summarised in Table 3. The results show that the dependence of final pH on concentration is significant but too small to be relevant.

DISCUSSION

The experimental data for the extraction of haem during acetone precipitation of myoglobin from phosphate buffer show similar trends to those for the precipitation of haemoglobin. The data also indicate a connection between the production of orthophosphoric acid and yield of haem. It is suggested that the cause of the release of haem may be a change in pH just before, or during, precipitation. It is not possible to comment on the microheterogeneous environment existing in the vicinity of the protein molecules as the transition from soluble to insoluble protein is made. These difficulties render a more detailed appraisal of the release of haem impossible at this stage.

Myoglobin and haemoglobin behave as acids and bases and any acid which is introduced into a solution of these proteins will serve to protonate them. The final pH of such a protein solution should therefore depend on the amount of acid introduced and the amount of protein present. In the case of mixtures of native myoglobin or haemoglobin with an acid such as orthophosphoric acid, the pH of the mixture should be higher the larger the amount of protein. The absence of a sufficiently large effect of concentration of myoglobin on the pH of the extracts shown in Table 3 demonstrates that it is not possible to immediately explain the effect of concentration of haemoprotein on the yield of haem in this way.

The reason for the production of orthophosphoric acid on addition of acetone to phosphate buffer is unknown but could be speculated to be due to an effect of the different solubilities of the various phosphate species in aqueous acetone. The dissociation of orthophosphoric acid is a dynamic equilibrium as follows:

$$H_3PO_4 \rightleftharpoons H_2PO_4^- + H^+$$
$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$$
$$HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+$$

which is established very rapidly. At pH 5·7 the predominating ionic form is $H_2PO_4^-$ but small amounts of the other ionic forms will always be present in equilibrium. The addition of acetone will cause one or more of the less soluble components to precipitate, thereby disturbing the equilibrium and causing further production of the insoluble components. If such an insoluble component is sodium monohydrogen orthophosphate or sodium orthophosphate then addition of acetone to a solution of sodium dihydrogen orthophosphate will cause the liberation of hydrogen ion. This, in turn, will convert some dihydrogen orthophosphate ion into orthophosphoric acid, which is expected to be the most soluble phosphate species in aqueous acetone.

Irrespective of the detailed explanation of the data, the results indicate the need for caution when using acetone precipitation and measurement of the amount of released haem for the determination of nitrosylhaemoproteins or free haem in the presence of myoglobin or haemoglobin. There are, however, some wider reaching implications of our results. Solvent precipitation of proteins from buffered solution is common practice, as is the production of 'acetone powders' by extraction of plant or animal tissues with acetone during the preparation of proteins, including enzymes, from these sources. The possibility that the protein is modified by a change of pH may be relevant in some of these cases.

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