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Acetone Extraction of Haem from Haemoglobin in the Presence of Acetate, Citrate and Phosphate Buffers

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ABSTRACT

The effects of components of acetate, citrate and phosphate buffers on the dissociation of haem from haemoglobin, during precipitation of haemoglobin from aqueous solution with acetone, are described. Unlike acetate or citrate, phosphate buffer appears to exert a considerable destabilising effect on haemoglobin, causing the liberation of haem; the yield of haem also increases as pH—and the concentration of haemoglobin—are reduced. The results have important implications for the determination, by acetone precipitation and the conversion of liberated haem to acid haematin, of nitrosylhaemoglobin in meat and model systems containing phosphate ion. Errors in determination may also arise from the adsorption of haematin on to filter paper when acetone extracts are filtered. An empirical correction for this effect is described.

INTRODUCTION

A frequently used method for the determination of nitrosylhaemoglobin and nitrosylmyoglobin in the presence of haemoglobin and myoglobin involves precipitation of the proteins with 80% acetone (aqueous) followed by acidification of the supernatant and spectrophotometric determination of the resulting acid haematin at 512, 540 or 640 nm (Hornsey, 1956; Gantner, 1960). The success of this method depends on the apparent lability of the nitrosylhaem prosthetic group under acetone precipitation conditions, in contrast to the stabilities of haemoglobin and

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myoglobin under the same conditions. The amounts of haem released after the precipitation of methaemoglobin and metmyoglobin from solutions at pH 5·5 are, respectively, 1% and 5% of the total haem present in the proteins (Fronticelli & Bucci, 1963). The yield of haem decreases with increasing pH. This procedure has been applied to studies of the conversion of haemoproteins to nitrosylhaemoproteins in cured meats (Walters *et al.*, 1968) and in model systems containing haemoprotein, nitrite ion and reducing agent (Fujimaki *et al.*, 1975). Acetone precipitation has also been used in studies of the dissociation of haem from the haemoproteins as a function of pH (Fronticelli & Bucci, 1963).

In recent work on the formation of nitrosylhaemoproteins in model systems buffered with phosphate ion and analysed by acetone precipitation and acidification, we noticed that the unreacted haemoproteins were themselves leading to the production of considerable amounts of a substance which was measurable as acid haematin. This effect was not apparent when phosphate ion was absent and the observation prompted us to investigate the effects of buffer composition on the release of free haem when haemoproteins are treated with aqueous acetone. The results of our investigation on the behaviour of haemoglobin are reported here.

EXPERIMENTAL

Acetate, citrate and phosphate buffers were prepared using AnalaR reagents and doubly distilled water and used within 2 days of preparation. Porcine haemoglobin was obtained from Sigma Chemicals Ltd (at least 75% oxidised, 2 × crystallised, dialysed and lyophilised). Solutions of haemoglobin were prepared by placing solid haemoglobin in solvent and leaving to stand until most of the solid had dissolved. The mixture was centrifuged (15 min at 1500 × *g*) and the supernatant used as stock haemoglobin solution. The concentration of haemoglobin was determined by mixing aliquots (2 ml) of haemoglobin solution with acetone (8 ml) acidified with hydrochloric acid (0·2 ml) and the mixture left for 25 min with gentle stirring. The mixture was centrifuged (10 min at 1500 × *g*) and its absorbance measured at 540 nm. Concentrations were calculated from absorbance data at this wavelength obtained using standard solutions of haematin chloride (Sigma Chemicals Ltd) and the results confirmed by the cyanmethaemoglobin method (Tentori & Salvati, 1981). The stock haemoglobin solution was diluted with solvent as required.

The amount of haem liberated on the treatment of haemoglobin solutions with acetone was determined according to the procedure used by previous workers to measure nitrosylhaemoproteins in meat and model systems (Hornsey, 1956; Gantner, 1960; Fujimaki *et al.*, 1975). Aliquots (2 ml) of haemoglobin solution were mixed with acetone (8 ml) and the mixture left for 25 min with gentle stirring before it was filtered through Whatman No. 4 filter paper (11 cm). The filtrate was acidified with hydrochloric acid (0·2 ml) and made up to 10 ml with aqueous acetone. The mixture was centrifuged (10 min at $1500 \times g$) and the absorbance of the resulting solution was measured at 540 nm. The concentration of acid haematin in this solution was calculated from appropriate calibration data as described above. In all cases the dilution factor, arising from the filtration and making up to 10 ml, of extracts, was determined and used to calculate the apparent concentration of haematin in the acetone extract of the protein.

RESULTS

The procedure for the determination of haem in the aqueous acetone extract involves a filtration step followed by dilution of the filtrate with acid and the addition of solvent to constant volume. Possible sources of error include loss of solution on the filter paper and glassware and adsorption, of liberated haem, on to the filter paper. Preliminary experiments involving thirty-six determinations showed that the volume of solution (in 80% acetone) held on the filter paper and apparatus was $1{\cdot}06 \pm 0{\cdot}14$ ml (the error is the standard deviation). When 10 ml of extract were filtered, and the filtrate made up to 10 ml, the corresponding dilution factor was therefore $1{\cdot}12 \pm 0{\cdot}02$. The result shows an error in the region of $\pm 2\%$ of the mean value when concentrations of haematin are determined in such filtrates and corrected for the dilution factor. Experiments to test for possible adsorption effects on to the paper showed that the concentration of haematin in the filtrate (before dilution) was always less than that in the original solution. The apparent loss of haematin was found to be dependent on its concentration. For example, when a solution containing 58 mg litre^{-1} of haematin chloride in 80% acetone was filtered, the filtrate contained haematin at a concentration of 91·8% of that of the original solution. The corresponding yield for a solution containing 1·5 mg litre^{-1} haematin chloride was 86·9%. These values

represented the limits of concentration of haematin found in the majority of the determinations carried out in this work. An empirical equation which allowed the correction of experimentally determined haematin concentrations, in this range, for adsorption effects was:

$$\frac{[Hm]}{[Hm]_o} \times 100 = 92 - \exp(-0{\cdot}0668[Hm] + 1{\cdot}68)$$

where $[Hm]$ is the concentration (in mg litre^{-1}) of haematin in the filtrate and $[Hm]_o$ is the concentration (in mg litre^{-1}) of haematin in the original solution or extract. It was not possible to reliably test the applicability of this equation when trace amounts (< 1 mg litre^{-1}) of haematin were present since the errors in determination were of similar magnitude to the results themselves. It is evident, however, that the loss due to adsorption when the most dilute solution referred to in this paper was analysed (approximately 0·3 mg litre^{-1}) does not exceed 50%.

All concentrations of haematin reported in this paper are corrected for the effects of dilution and for adsorption, with the exception of instances where the solution contained less than 1·5 mg litre^{-1}. The concentrations and recoveries refer, therefore, to those in the acetone extract of haemoglobin. Since, in all cases, the haemoglobin undergoes a fivefold dilution on the addition of acetone, the equivalent amount of haematin, in the form of haemoglobin, in the original haemoglobin solution, is five times the value shown, neglecting changes in volume when acetone and water are mixed.

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 mixtures and the effects of all three buffer systems were, therefore, considered. 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, chosen to be in the range pH 5·7 to 5·8, although, at these values, neither the acetate nor the phosphate buffers have much buffering capacity. The pH values reported are measured values for the final haemoprotein solution. The ability of the components of phosphate buffer to promote the dissociation of the haemoprotein is clearly demonstrated in Table 1 where each value is the mean of six separate determinations and the errors are standard deviations. The total amount of haem in a solution of haemoglobin

TABLE 1
The Effect of Components of Acetate, Citrate and Phosphate Buffers on Release of Haem from Haemoglobin 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	5·74	0·10	0·7 ± 0·1	44·7 ± 0·4
Citrate	5·71	0·09	2·0 ± 0·2	45·6 ± 0·4
Phosphate	5·77	0·10	38·1 ± 4·7	44·3 ± 0·1

analysed by the acid haematin and cyanmethaemoglobin methods agreed to within 4%.

The extent to which the components of phosphate buffer cause dissociation of haemoglobin, shown in Table 1, was determined from absorbances at 540 nm, irrespective of whether the chromophore was acid haematin. The visible spectra of acetone extracts of haemoglobin in phosphate buffer, before and after acidification, are shown in Fig. 1. The spectrum of the extract before acidifying is one with indefinite peaks, as is shown to occur for solutions of haematin chloride (Lewis, 1954). On acidifying, a characteristic spectrum of acid haematin with peaks at 512, 540 and 640 nm is obtained (Hornsey, 1956). Both samples also show an intense soret peak (385 and 400 nm for acidified and non-acidified extracts, respectively) in the ultraviolet, confirming further the presence of the haem moiety. It is concluded, therefore, that the increase in

TABLE 2
The Effect of pH on the Release of Haem from Haemoglobin in 0·1 M Phosphate Buffer, when Haemoglobin is Precipitated in 80% Acetone (Aqueous)
(Concentrations determined as acid haematin in acetone extract)

pH	*Per cent extraction*	*Total haematin* ($mg\ litre^{-1}$)
5·69	39·3 ± 2·6	54·0 ± 0·1
5·87	30·1 ± 1·3	53·5 ± 0·3
6·27	8·7 ± 1·5	53·1 ± 0·4

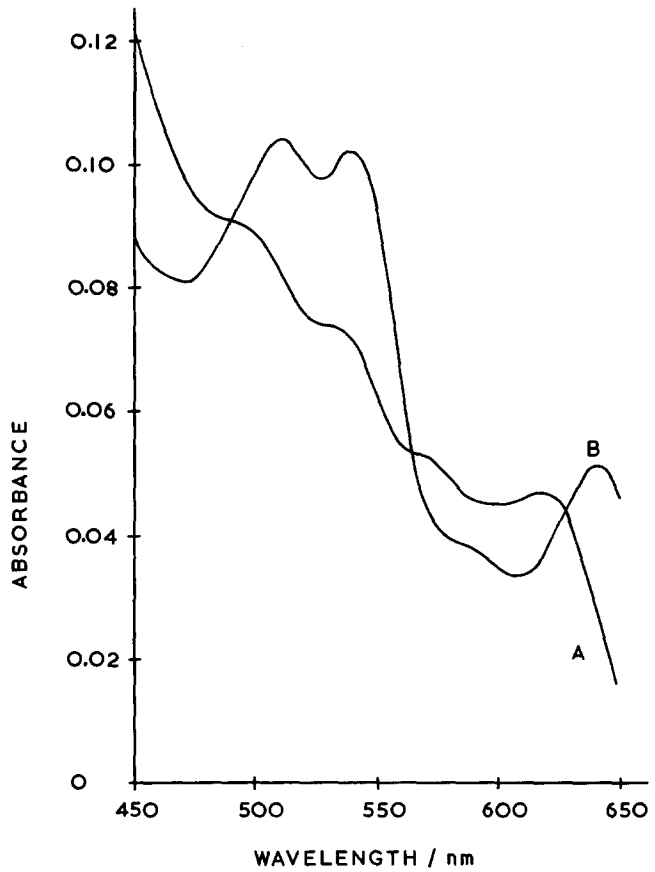

Fig. 1. Visible spectrum of acetone extract of haemoglobin buffered with phosphate at pH 6·0. A: Before acidifying. B: After acidifying.

absorbance at 540 nm, when haemoglobin in phosphate buffer is precipitated with acetone and the extract is acidified, is due to dissociation of haem from the haemoprotein.

When considering the importance of these observations to the analysis of nitrosylhaemoglobin or free haem in the presence of haemoglobin, it is also desirable to know the effects of pH and of concentration of buffer and haemoglobin. The effect of pH is reported in Table 2. The yield of haem appears to be sensitive to pH, higher yields being obtained at lower pH values. In separate experiments where the buffers contained either a constant dihydrogen orthophosphate ion concentration and variable monohydrogen orthophosphate ion concentration or a constant total

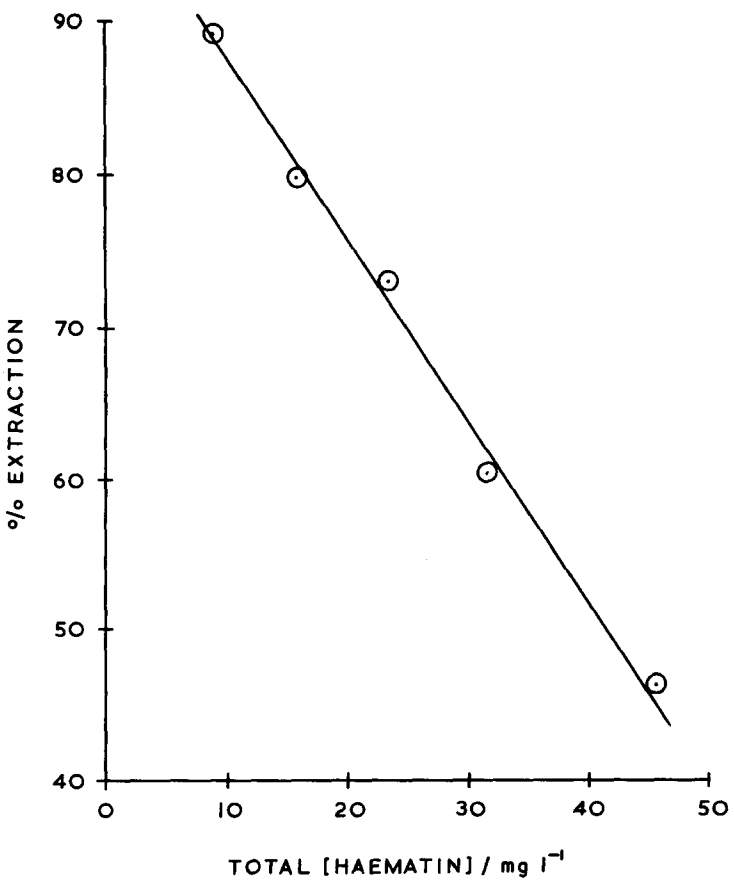

Fig. 2. The effect of concentration of haemoglobin on the extent to which haem is extracted on precipitation with acetone. Buffer: 0·1 M phosphate, pH 5·56. Concentrations determined as acid haematin in acetone extract.

phosphate species concentration but with variable pH, no specific effects of either ion could be detected. This result indicates that the effect of pH is a specific effect of hydrogen ions on the process of dissociation and not merely a reflection of differing buffer composition. The mechanism of dissociation therefore involves phosphate ion in general and, from additional experiments, the process is found to depend on buffer concentration, the extent of dissociation increasing with increasing concentration. The effect of haemoglobin concentration on the yield of haem is shown graphically in Fig. 2. It is evident that low concentrations of the haemoprotein favour the liberation of haem, the relationship between the

proportion of haem liberated and the concentration of haemoglobin being linear over the concentration range chosen (9–46 mg litre^{-1} haematin in the acetone extract).

In order to test for any specific interaction between haemoglobin and the components of phosphate and acetate buffers, the visible spectra of solutions of the haemoprotein in these buffers were recorded over a period of 2 h. No spectroscopic differences could be detected.

In preliminary experiments, the findings reported here have been found to apply to haemoglobin from other sources (sheep and bovine) although the magnitudes of the various effects are different. Preliminary experiments have also been carried out to test the behaviour of metmyoglobin under similar conditions. The results show the same trends as observed for haemoglobin.

DISCUSSION

The experimental data demonstrate that phosphate species greatly enhance the liberation of haem when haemoglobin is precipitated from aqueous solution with acetone. It is unlikely that this effect is one of ionic strength since the ionic strengths of 0·1 M acetate and phosphate buffers at pH 5·7–5·8 are expected to be very similar. It is therefore a specific ion effect, the only other species known to exert such a haem-labilising effect being nitric oxide. The latter adds to the iron of the haem as a ligand, causing a considerable change in the spectrum of the haem group. In contrast, there is no change in the spectrum of haemoglobin when phosphate buffer is replaced by acetate buffer, even though these buffers show very different haem-labilising properties on precipitation with acetone. There is, therefore, no evidence to suggest that nitric oxide and phosphate species exert their effects by similar mechanisms and it is probably more appropriate to consider interactions between phosphate species and the whole protein molecule. Interactions between haemoglobin and organic and inorganic phosphates are known to occur and to modify the ability of the haemoprotein to bind oxygen (Gibson, 1970) or to substantially decrease the rate of recombination of carbon monoxyhaem with apohaemoglobin (Chu & Bucci, 1979). The latter resulted from conformational changes to the apohaemoglobin. An alternative possibility is that solvation of haem in aqueous acetone could be made more favourable by interaction between the free haem and phosphate ion.

The most important direct consequences of this work relate to the determination of free haem or nitrosylhaemoglobin in the presence of haemoglobin when phosphate buffers are used. Preliminary work with metmyoglobin shows that the results can be extended qualitatively to this pigment and analytical data obtained in the presence of phosphate ion need to be re-examined. A further source of error which has been identified is the adsorption of haematin on to filter paper when aqueous acetone extracts are filtered. The results may easily be corrected for this effect. It need not, however, reflect the behaviour of the nitrosyl-pigment and further investigation of this is required.

The haemoglobin used in this work was mainly in the oxidised form but it is likely that, in model reactions and meat systems, reduced forms of the pigment and oxyhaemoglobin will be present. Further work is required to assess the stability of these species under acetone-precipitation conditions.

REFERENCES

Chu, A. H. & Bucci, E. J. (1979). Effect of polyanions on the kinetics of the reaction of apohaemoglobin with carbon monoxyhaem. *J. Biol. Chem.*, **254**, 3772–6.

Fronticelli, C. & Bucci, E. (1963). Acetone extraction of haem from myoglobin and haemoglobin at acid pH. *Biochim. Biophys. Acta*, **78**, 530–1.

Fujimaki, M., Emi, M. & Okitani, A. (1975). Fate of nitrite in meat-curing model systems composed of myoglobin, nitrite and ascorbate. *Agric. Biol. Chem.*, **39**, 371–7.

Gantner, G. (1960). Zur bestimmung der farbe von gepökeltem fleisch und fleischerzeugnissen. *Z. Lebensm. Unters. Forsch.*, **111**, 277–81.

Gibson, Q. H. (1970). Organic phosphate and ligand binding in haemoglobin. *Biochem. Biophys. Res. Comm.*, **40**, 1319–24.

Hornsey, H. C. (1956). The colour of the cooked cured pork. I. Estimation of the nitric oxide-haem pigments. *J. Sci. Food Agric.*, **7**, 534–40.

Lewis, U. J. (1954). Acid cleavage of haem proteins. *J. Biol. Chem.*, **206**, 109–20.

Tentori, L. & Salvati, A. M. (1981). Haemoglobinometry in human blood. In: *Haemoglobins. Vol. 76 of Methods in enzymology* (Colowick, S. P. & Kaplan, N. O. (Eds)), Academic Press, New York, 707–15.

Walters, C. L., Taylor, A. M. McM., Casselden, R. J. & Ray, N. (1968). *Investigation of specific reducing systems in relation to meat curing.* British Food Manufacturers Research Association, Research Report 139, June, 1968, BFMIRA, Leatherhead, Surrey.