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Kinetics of Processes Involved in the Precipitation of Myoglobin with Acetone in the Presence of Phosphate Buffer

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ABSTRACT

When myoglobin in phosphate buffer is precipitated with acetone, a proportion of the available haem is released due to acidification of the medium. The factors which determine the yield of haem include the rates of change of pH, release of haem and aggregation of protein.

Kinetic measurements show that when acetone is mixed with buffered myoglobin (phosphate buffer, 0·1 M) the change in pH precedes other reactions which determine the yield of haem. The first-order rate constant for the release of haem from myoglobin in a medium prepared by mixing phosphate buffer (0·1 M, pH 5·7) with acetone (final acetone content 72%) is 2·8 s^{-1} at 24°C. The second-order rate constant for the aggregation of myoglobin in the same medium is estimated to be of the order of 10^9 M^{-1} s^{-1}. The relevance of these findings to an understanding of the factors underlying the extent of release of haem when myoglobin is precipitated from buffered solution with acetone is discussed.

INTRODUCTION

It has previously been shown (Wedzicha & Ladikos, 1985; 1986) that when myoglobin or haemoglobin are precipitated from aqueous solution with

acetone, the presence of components of phosphate buffer ($H_2PO_4^-$ and HPO_4^{2-} species) appears to lead to a considerable destabilising effect on the haemoprotein, causing the liberation of haem. It was suggested that this effect is caused by a fall in the pH of the medium when acetone is mixed with phosphate buffer and subsequent acid denaturation of haemoprotein which is accompanied by expulsion of haem (Wedzicha & Ladikos, 1986). The fall in pH is likely to be a result of selective precipitation of the more basic phosphate species. It was also found that the yield of haem increases linearly with decrease in concentration of haemoprotein but this effect could not be simply related to an effect of pH.

It is expected that the amount of haem formed in the system in question is dependent on the relative rates of three processes:

1. The change in pH of the medium when aqueous phosphate buffer is mixed with acetone.
2. Acid promoted liberation of haem from haemoprotein.
3. Aggregation and precipitation of the protein.

The purpose of this investigation is to obtain an estimate of the rates of these processes in appropriate solvent mixtures to use later in models of the mechanism of the precipitation process in order to reconcile the effect of concentration of haemoprotein on yield of haem.

EXPERIMENTAL

Preparation of reagents

The myoglobin used was sperm whale skeletal muscle myoglobin (Sigma Chemicals Ltd), described as essentially salt-free, 95–100%, crystallised and lyophilised. The purity of this sample was assessed in detail previously (Wedzicha & Ladikos, 1986) and solutions of the haemoprotein prepared as before. Wherever possible, AnalaR grade reagents were used. Phosphate buffer was prepared using the sodium salts of HPO_4^{2-} and $H_2PO_4^-$ species.

The ideal reaction medium for following the kinetics of the processes involved in precipitation of myoglobin is a mixture of buffered myoglobin and acetone. Unfortunately, the phosphate salts precipitate rapidly, giving rise to turbidity which prevents spectrophotometric or light scattering experiments involving the other components of the system. For such measurements a solvent which had the composition of the solution remaining after mixing phosphate buffer with acetone was used. For this purpose, phosphate buffer (0·1 M, pH 5·7) was mixed with acetone to give a final composition of 80% acetone, 20% water (v/v), the mixture centrifuged

(5700 × *g*, 10 min) and the supernatant used as the acetone-containing component of reaction mixtures. All reagents were allowed to come to thermal equilibrium at 24°C prior to reaction.

Effect of myoglobin concentration on yield of haem

In order to investigate the effect of myoglobin concentration on the yield of haem in the acetone–phosphate buffer solvent, aliquots (1 ml) of a stock aqueous myoglobin solution were mixed with solvent (9 ml) and the mixture left for 25 min with gentle stirring. The precipitate was removed by centrifugation (1500 × *g*, 10 min). An aliquot (5 ml) of the supernatant was acidified with HCl (0·1 ml) and the volume made up to 10 ml with acetone and water to give a final composition of 80% acetone and 20% water (v/v). The absorbance of the resulting solution was measured at 540 nm and the haem content determined as described previously (Wedzicha & Ladikos, 1985). The total amount of haem available for extraction was also determined by the acid haematin method described in the same paper.

Continuous flow kinetic measurements

The rate of pH change when phosphate buffer and acetone are mixed was studied by means of a continuous flow apparatus. For this purpose acetone and phosphate buffer (0·1 M, pH 5·7) were driven in a volume ratio of 4:1 through a three-way tap, serving as a mixing chamber, into an observation tube. Two indicators, a mixture of bromocresol green (0·075% w/v) and methyl red (0·05% w/v) (green to red) or bromophenol blue (blue to yellow), were added individually to the acetone phase to monitor the change in pH. The flow rate of reactants was measured by timing a known volume, and the volume of the mixing chamber found by weighing the amount of mercury required to fill it.

Stop-flow kinetic measurements

Stop-flow kinetic measurements were used to determine the rate of dissociation of haem from myoglobin in the aqueous acetone solvent. The apparatus consisted of an all-glass flow circuit with two drive syringes delivering reactants in a volume ratio of 1:9 (aqueous myoglobin:acetone–phosphate buffer solvent) into a three-way tap which served as a mixer. The reaction mixture was passed into a 50 μl flow cell in the cell compartment of a Cecil 292 spectrophotometer. The output of the instrument was passed through an amplifier with variable gain and offset, and the signal displayed on an oscilloscope. The flow of reactants was stopped by passing the mixture

from the flow cell into a stopping syringe which stopped against a microswitch used to trigger the oscilloscope.

The response of the spectrophotometer unit was determined by closing off the light source and replacing it with a light emitting diode (rise and fall times $<1\ \mu s$) which was driven by a square wave oscillator (400 ms period, rise and fall times $<100\ \mu s$). In all cases the signal displayed on the oscilloscope was recorded photographically.

The results of some of the kinetic measurements were confirmed by trial runs using a Hi-Tech Scientific SF51 stop-flow module connected to an SU40A spectrophotometer unit. The data were logged and processed on an Apple 2E microcomputer by means of an MCS1 data system and rate constants calculated using a least squares method based on that of Edsall & Gutfreund (1983).

Light scattering measurements

Light scattering was used to follow the kinetics of aggregation of myoglobin and its products of denaturation in the acetone–phosphate buffer solvent. Light of 633 nm from a 35-mW He–Ne laser (Spectra Physics Type 124B) was scattered from solutions contained in a square 1 cm fluorimeter cell which was placed in the sample compartment of a light scattering goniometer (Malvern PCS 100SM) with the photomultiplier connected to a Malvern 'Log-Lin' 7027 autocorrelator system. Photons scattered at an angle of 90° to the incident beam were counted over 1 s time intervals, each of which was followed by a delay of 2·43 s (to allow for computation) before the next counting episode was initiated. In a kinetic experiment, 100 consecutive counting episodes were performed for each coagulating sample.

To ensure that solutions were free from dust particles, all aqueous solutions (i.e. those containing myoglobin and those used to dilute the stock acetone) were filtered twice through cellulose nitrate filters of pore size 0·22 μm. The acetone-containing solutions could not be filtered. The reaction between the acetone–phosphate buffer solvent and myoglobin was started by placing a convenient known volume (1·5–2·5 ml) of solvent in the fluorimeter cell, adding water (0–1·0 ml) if necessary, followed by an aqueous solution of myoglobin (0·1–0·5 ml) of defined concentration. The contents of the cell were mixed by inverting twice, the cell was immediately placed in the light scattering equipment and photon counting was initiated. The time lapse between mixing and the first measurement was approximately 5 s. The concentrations of myoglobin used in the experiments depended on the acetone concentration; high concentrations of acetone made it necessary to slow down the reaction by decreasing the myoglobin concentration. The range of concentrations varied from 0·67 to $8{\cdot}4 \times 10^{-4}$ mg ml^{-1}.

The calibration of the instrument was carried out using a solution of a known concentration of myoglobin in phosphate buffer (0·1 M, pH 5·7) with the buffer as a blank.

RESULTS

Rate of pH change

The maximum rate of flow obtained in the continuous flow kinetic apparatus was 17 ml s^{-1} and it was judged that both indicators changed to their final colours within the first 10% of the volume of the mixing chamber. The volume of the chamber was found to be 24 μl and it is therefore estimated that the change in pH took place within a fraction of a millisecond. When bromophenol blue was mixed with the acetone–phosphate buffer solvent its colour (green) was intermediate between those of the acid and base forms of the indicator. A similar colour was seen in the continuous flow system, suggesting that the pH obtained after a fraction of a millisecond is similar to the final pH of the system. It is, of course, not possible to regard this pH value literally since the behaviour of the indicator in the acetone–water mixture is unknown. The result does, however, serve to illustrate the order of magnitude of the rate of pH change.

Rate of release of haem

The rate of expulsion of haem from haemoproteins during acid denaturation in aqueous solutions has been measured by following the change in the soret absorbance band of the haemoprotein (Polet & Steinhardt, 1969). Therefore, in this work the progress of the reaction was followed by measuring the rate of change of absorbance at specific wavelengths in the range 390–420 nm. In all experiments the concentration of myoglobin at the point of mixing was 1·2 μM. The time between mixing and the first measurement was estimated to be 92 ms and the subsequent change in absorbance was followed over periods of 50 ms to 2 s.

In order to test the reproducibility of the stopped flow device, 6–8 kinetic runs were carried out at each of three wavelengths (395, 400 and 405 nm). Relative absorbance data at three measurement times (zero, 200 and 500 ms) at each wavelength are given in Table 1. It is evident that, with the exception of one run, the coefficients of variation are below 5%.

The change in the soret band as the reaction time is increased from 92 to 1292 ms is shown in Fig. 1, together with the absorbance spectrum of the same concentration of hydroxyhaemin in the reaction medium. The spectra

TABLE 1
Reproducibility of Absorbance Measurements in Stop-flow Kinetic Experiments

Wavelength (nm)	*Relative absorbance at times shown time (ms)*		
	0	*200*	*500*
395	31·5	35·0	39·5
	30·0	33·0	36·5
	29·5	32·0	35·0
	32·0	35·5	39·5
	32·0	36·0	40·5
	32·5	36·0	40·5
	31·3 ± 1·2[a]	34·6 ± 1·7	35·6 ± 2·3
400	34·0	37·0	39·5
	35·5	38·0	42·0
	35·0	38·0	42·0
	36·6	39·0	43·0
	35·5	39·5	42·0
	37·5	42·0	44·0
	38·0	41·0	45·5
	36·0	40·5	44·0
	36·0 ± 1·3	39·4 ± 1·7	42·8 ± 1·8
405	39·0	41·0	42·5
	40·0	42·0	44·5
	40·0	41·0	44·0
	40·0	43·0	45·5
	40·5	44·0	45·5
	41·5	44·5	47·5
	40·2 ± 0·8	42·6 ± 1·5	44·9 ± 1·7

[a] Mean ± standard deviation shown for each set of measurements.

obtained for reaction mixtures show an isosbestic point at 410 nm, which suggests that the observed change is the quantitative conversion of one absorbing species to another. The observation that the spectrum of hydroxyhaemin also passes close to the isosbestic point confirms that one of these species is haem with $\lambda_{max} = 402$ nm. The other species will be haemoprotein, although not necessarily in its native state.

Previous workers (Polet & Steinhardt, 1969) observed that the release of haem from myoglobin in aqueous solution is a first-order process and it is, therefore, reasonable to analyse the data obtained here in terms of first-order kinetics. Examples of first-order plots ($\ln(A_\infty - A_t)$ against time, where A_t and A_∞ are, respectively, the absorbances at any wavelength at

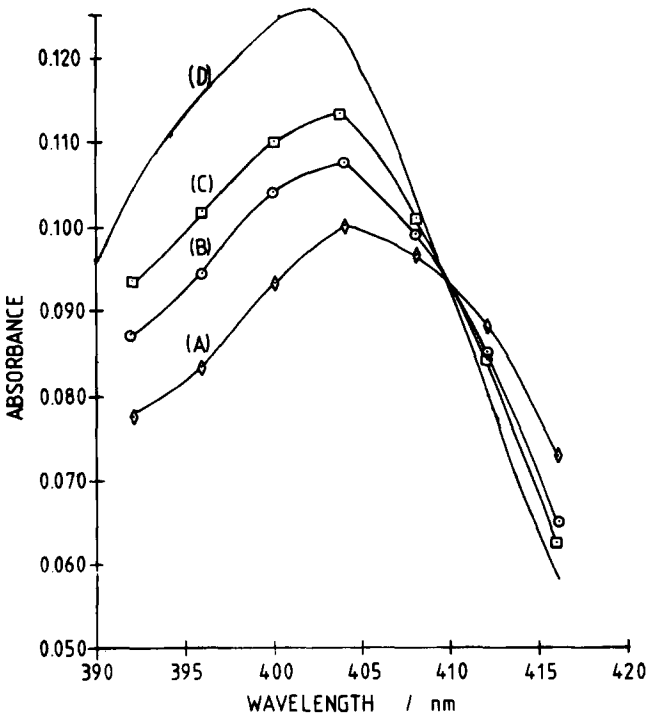

Fig. 1. Changes in the soret absorbance band during reaction of myoglobin in acetone–phosphate buffer solvent. Initial conditions: acetone content, 72%; myoglobin concentration, 1·2 μM; original buffer, 0·1 M, pH 5·7; 24°C. (A) 92 ms after mixing; (B) 492 ms after mixing; (C) 1292 ms after mixing; (D) spectrum of hydroxyhaemin (1·2 μM).

times t and ∞) at 392 and 416 nm are shown in Fig. 2 and give first-order rate constants of 2·83 and 3·65 s^{-1} for formation of haem and loss of haemoprotein, respectively.

Measurement of the response of the spectrophotometer used for these kinetic experiments showed that its time constant was equivalent to a first-order process with a rate constant of 17 s^{-1}. The values measured for the release of haem are therefore not seriously affected by the response of the instrument.

The main shortcoming of the results presented so far is the long 'dead-time' between mixing of reactants and the first measurement. Since this could conceal a fast change, a stop-flow experiment with a dead-time <1 ms was carried out. First-order rate constants of, respectively, 2·85 and 2·98 s^{-1} for formation of haem and loss of haemoprotein were found to apply at all reaction times between 1 ms and 2 s. The agreement between rate constants measured using the two sets of stop-flow apparatus gives added confidence in the results whilst the similarity in value for the two processes further supports the assumption that only two processes are being observed.

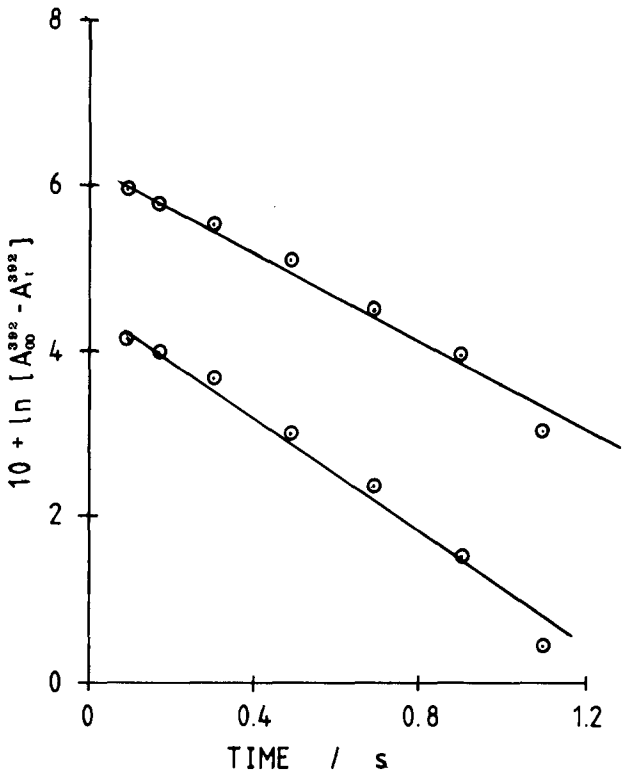

Fig. 2. First-order plots of change in absorbance at 392 and 416 nm from stop-flow kinetic experiments. Initial conditions: acetone content, 72%; myoglobin concentration, 1·2 μM; original buffer, 0·1 M, pH 5·7; 24°C. (A) 392 nm; (B) 416 nm.

It is always important not to lose sight of the possibility that there could be an even faster reaction which is not being observed. Unfortunately, it is not possible to observe the absorbance spectrum of the haemoprotein under reaction conditions and the likelihood of such a reaction cannot be assessed here. If such a reaction exists, then it is essentially complete within 1 ms of mixing. If it is assumed that at least five half-lives must have elapsed within this period, the minimum value of rate constant is of the order of $3{\cdot}5 \times 10^3\ s^{-1}$.

Rate of aggregation

The calculation of the rate constant for the aggregation of the protein was based on the assumption that the weight-average molecular mass ($\bar{M}_w$) of the protein aggregate increases linearly with time, such behaviour being typical of Smoluchowski kinetics (Parker & Dalgleish, 1977). According to this model

$$\bar{M}_w = M_0(1 + kCt) \quad (1)$$

where M_0 is the molecular mass of the monomer, C is the concentration and k is the rate constant for aggregation. The intensity $I(\theta)$ of light scattered at an angle θ from a solution of macromolecules is simply given by

$$I(\theta) = \bar{M}_w KCP(\theta) \tag{2}$$

where K is an optical constant depending on the geometry of the measurement system and the refractive properties of solvent and solute, and $P(\theta)$ is the particle scattering factor. For small particles whose dimensions are less than about one-twentieth of the wavelength of the incident light, $P(\theta) = 1$ for all angles and we assume that it has this value in our experiments. The value of the quantity KM_0 for this particular system was determined from the intensity of scattered light (number of scattered photons detected per second) for an aqueous solution of myoglobin ($M_0 =$ 17 816 daltons; Edmundson & Hirs, 1961) of known concentration and was found to be 804 litres $g^{-1} s^{-1}$.

From eqn (1) and the relationship between light scattering intensity and molecular mass, it is clear that in an individual experiment the rate constant for aggregation can be found from the slope of the plot of intensity against time:

$$k = \frac{17\,816}{804C^2} \frac{dI_t}{dt} \tag{3}$$

where the value of k is in $M^{-1} s^{-1}$ and C is in g litre^{-1}. Figure 3 shows a typical plot of scattered intensity as a function of time at a haemoprotein concentration of 1·7 μg ml^{-1} (95 nM). In practice the rate constant for the aggregation was measured from the slope of the graphs at $t = 0$. Most experiments were carried out in duplicate and the results of all measurements are shown in Fig. 4 to illustrate the effect of solvent composition on the rate constant. It is evident that the value of k increases by nearly six orders of magnitude as the acetone content of the medium is increased from 40% to 77% (v/v) and rate constants of the order of $10^9 M^{-1} s^{-1}$ are observed when the acetone content is >72%.

A possible source of error lies in the use of aqueous solutions of myoglobin for calibration of the scattering system and of acetone–phosphate buffer solutions in kinetic measurements, because the refractive indices (and hence light scattering properties) of the two solvents differ. Measurement of the refractive indices of the two solvents gave, respectively, values of 1·336 and 1·366. This difference is not likely to cause significant alterations in the results or their interpretation, especially since greater sources of error exist. These arise from the relatively slow mixing of the solutions before measurements are started, and the increasing uncertainty of the value of $P(\theta)$ as the sizes of the aggregates increase. Since the rate of a

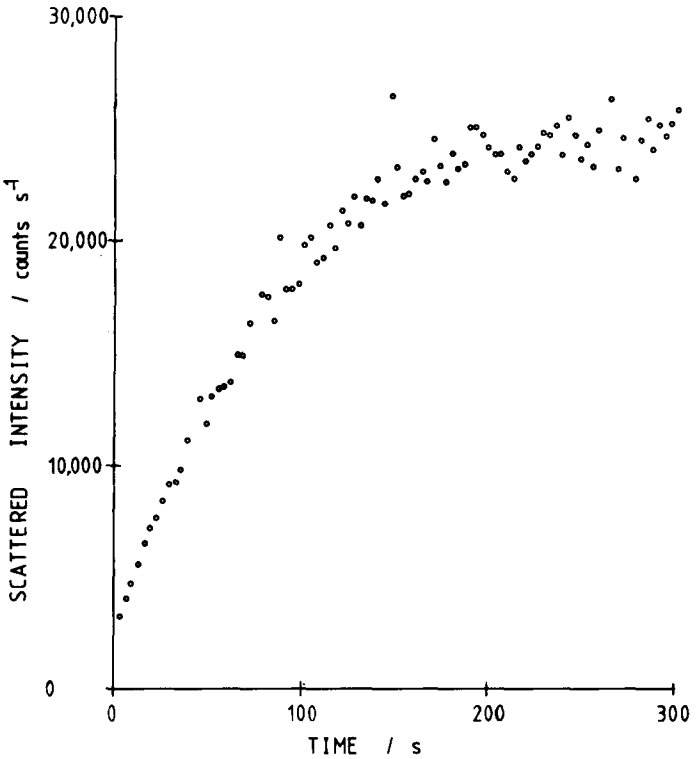

Fig. 3. Graph of scattered intensity as a function of time for myoglobin in acetone–phosphate buffer solvent. Initial conditions: acetone content, 77·3%; myoglobin concentration, 95 nM; original buffer, 0·1 M, pH 5·7; 24°C.

second-order process is sensitive to concentration, the variable concentration gradients during mixing of reagents could lead to irreproducibility, although the results in Fig. 4 show little evidence that such a problem exists in practice. The theory outlined above predicts that graphs of scattered intensity with time should be linear. This is true for the initial portions of the plots, but, as Fig. 3 shows, there is a drop-off in the apparent rate at long times. This will almost certainly be caused by the decrease in $P(\theta)$ from its initial value of 1 as the sizes of the aggregate particles increase. There may also be some sedimentation of the largest aggregate particles as the reaction proceeds, for which no correction can be made.

It was hoped that the linear initial phase of the measurements was free from these sources of error, although at the highest concentrations of acetone the starting point of the measurements represents aggregates with average molecular masses of the order of $1500 \times M_0$ and the rate constants were calculated assuming that the linear plot can be extrapolated through the initial 'dead-time' in which the mixing takes place to a value of M_0 at the

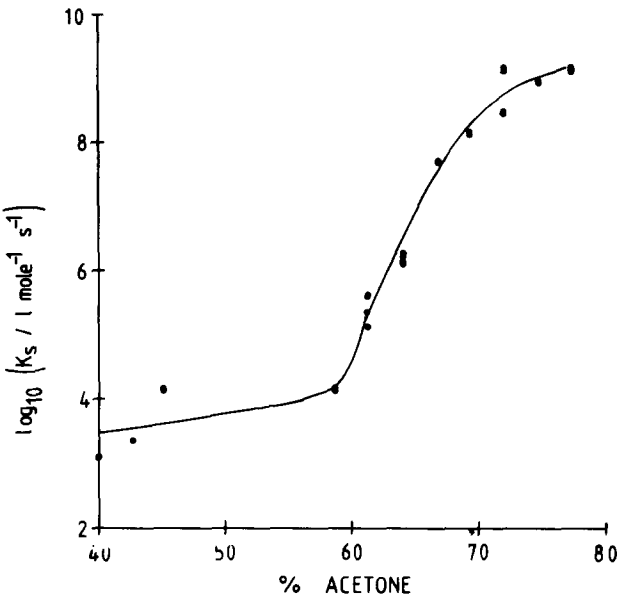

Fig. 4. Variation of second-order rate constant for aggregation of myoglobin as a function of acetone content in acetone–phosphate buffer solvent.

instant of mixing. Because of the uncertainties, the values of the rate constants shown in Fig. 4 should be regarded only as order of magnitude estimates. It is interesting to note that the highest value of rate constant observed is similar to that reported for a diffusion-controlled aggregation in water (*c.* $10^9\ M^{-1}\ s^{-1}$; Chang, 1981) and it is encouraging that our values begin to level off at this magnitude, when the acetone concentration is high.

Yield of haem on precipitation of myoglobin

In all previous work on the release of haem during precipitation of haemoproteins with acetone (Wedzicha & Ladikos, 1985, 1986), acetone was added to the haemoprotein buffered to pH > 5·7 with phosphate species. In order to avoid the formation of an inorganic precipitate in this work, unbuffered myoglobin was added to acetone–phosphate buffer mixture which was free from suspended solids. The final acetone concentration was 72% (v/v), whilst the effect of concentration of myoglobin on the yield of haem has previously been investigated at a final acetone content of 80% (v/v). It is important, therefore, to repeat the previous work with the solvent system used here for kinetic studies, particularly since no accurate temperature control had been used previously. The results are summarised in Fig. 5, where each point is the mean of four determinations and the error bars are standard deviations. Whilst the relationship between yield and

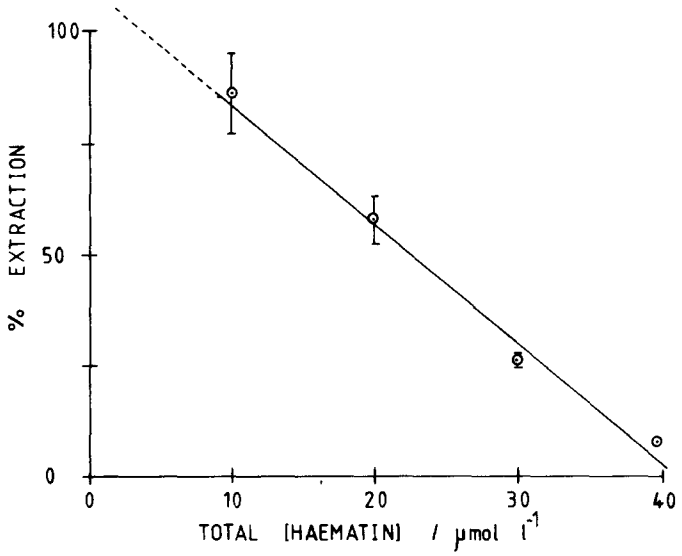

Fig. 5. Effect of concentration of myoglobin on yield of haem when myoglobin solution is added to acetone–phosphate buffer solvent. Initial conditions: acetone content, 72%; original buffer, 0·1 M, pH 5·7; 24°C. The concentration of myoglobin is the initial concentration in the reaction mixture.

myoglobin concentration is still essentially linear, the effect observed here is much more sensitive to changes in myoglobin concentration than found previously. Current data fit the equation

$$\%\,\text{yield} = 111{\cdot}3 - 2{\cdot}7 \times 10^6[\text{myoglobin}] \quad (4)$$

whilst the corresponding relationship from previous experiments involving precipitation of myoglobin from buffered solutions is

$$\%\,\text{yield} = 104{\cdot}5 - 0{\cdot}76 \times 10^6[\text{myoglobin}] \quad (5)$$

DISCUSSION

It is clear from the results presented here that when acetone is added to phosphate buffer there is a rapid fall in pH. If myoglobin is present in solution the protein will begin to denature, releasing haem, and the myoglobin and globin will aggregate. Typical concentrations of haemoproteins used in experiments involving precipitation from aqueous buffer (Wedzicha & Ladikos, 1985, 1986) are in the range 10–50 μM. If the release of haem is considered to be a first-order process with a rate constant of 2·8 s^{-1}, its half-life is of the order of 240 ms and is independent of concentration. It

appears that the change in pH could be at least three orders of magnitude faster than the rate of release of haem. If the amount of haem produced after precipitation of the protein is a result of aggregation competing with dissociation, then the apparent rates of the processes must be comparable for there to be a significant change in yield of haem with concentration of myoglobin. Given a second-order rate constant of $10^9\,\text{M}^{-1}\,\text{s}^{-1}$ for the aggregation process, eqn (1) may be used to predict that, on the time scale of the release of haem, aggregates with average masses of the order $10^3 \times M_0 - 10^4 \times M_0$ are formed. There will, of course, be a wide distribution of masses and much larger aggregates will exist with the likelihood of sedimentation taking place. Supplementary experiments have shown that, when myoglobin is precipitated with acetone from aqueous solution without phosphate buffer, the solid will not release haem when suspended in the acetone–phosphate buffer medium (Ladikos, 1986).

It is interesting to compare the different yields of haem when myoglobin is precipitated from phosphate buffer with acetone and when the protein is precipitated from the acetone–phosphate buffer medium used in this work. The results indicate that the yield of haem is generally higher in the system where phosphate salts precipitate at the same time as the protein, particularly at high haemoprotein concentration. In both types of experiment the rate of change of pH is expected to be fast on the time scale of the release of haem or formation of sufficiently large protein aggregates and it is unlikely that the difference in mechanism of pH change is the determining factor. Apart from the presence of inorganic precipitate, the addition of acetone to myoglobin in phosphate buffer will give rise to reaction mixtures with compositions very similar to those investigated here and, therefore, the differences in behaviour could arise from (i) additional effects of precipitated phosphate species and (ii) transient effects when acetone is added to the buffered haemoprotein; the mixing time is, of course, long when compared to the half-lives of the individual processes taking place.

Whilst it is recognised that the data obtained here are being compared with previously reported values where no temperature control had been exercised, it is considered that the effects of temperature are small in comparison with the observed differences. For example, an increase in temperature from 16·4–24·0°C causes an increase in yield from 67% to 77% when myoglobin is precipitated from 0·1 M phosphate buffer (initial myoglobin concentration = 35 μM), pH 5·7 (Ladikos, 1986). In the work reported here the corresponding yield was of the order of 10%.

It is now proposed to use the rate constants obtained here in a kinetic model of the precipitation process. In particular, it is necessary to deduce whether the individual processes considered here can be treated in isolation

when acetone is added to myoglobin in phosphate buffer or whether they interact with one another. A detailed analysis of the system also requires that the causes of each stage in precipitation be known. Whilst the cause of release of haem is undoubtedly the change in pH, it is not clear whether the rate of precipitation of protein is affected by the pH of the medium. Further work on these lines is required.

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