Kinetics of β-haematin formation from suspensions of haematin in aqueous benzoic acid

Timothy J. Egan* and Mmboneni G. Tshivhase

Received 28th July 2006, Accepted 24th August 2006
First published as an Advance Article on the web 31st August 2006
DOI: 10.1039/b610866k

Kinetics of β-haematin (synthetic malaria pigment) formation from haematin have been studied in the presence of aqueous benzoic acid and derivatives of benzoic acid. Formation of the β-haematin product is demonstrated by X-ray diffraction and IR spectroscopy. Reactions were followed by determining the fraction of unreacted haematin at various time points during the process *via* reaction of extracted aliquots with pyridine. The kinetics can be fitted to the Avrami equation, indicating that the process involves nucleation and growth. Reaction kinetics in stirred benzoic acid are similar to those previously observed in acetic acid, except that benzoic acid is far more active in promoting the reaction than acetic acid. The reaction reaches completion within 2 h in the presence of 0.050 M benzoic acid (pH 4.5, 60 °C). This compares with 1 h in the presence of 4.5 M acetic acid and 4 h in the presence of 2 M acetic acid. The reaction rate in benzoic acid is not affected if the stirring rate is decreased to zero, but very vigorous stirring appears to disrupt nucleation. The rate constant for β-haematin formation in benzoic acid has a linear dependence on benzoic acid concentration and follows Arrhenius behaviour with temperature. There is a bell-shaped dependence on pH. This suggests that the haematin species in which one propionate group is protonated and the other is deprotonated is optimal for β-haematin formation. When the reaction is conducted in *para*-substituted benzoic acid derivatives, the log of the rate constant increases linearly with the Hammett constant. These findings suggest that the role of the carboxylic acid may be to disrupt hydrogen bonding and π-stacking in haematin, facilitating conversion to β-haematin. The large activation energy for conversion of precipitated haematin to β-haematin suggests that the reaction *in vivo* most likely involves direct nucleation from solution and probably does not occur in aqueous medium.

Introduction

Haemozoin is a microcrystalline cyclic dimer of ferriprotoporphyrin IX (Fe(III)PPIX) in which the propionate side chain of one protoporphyrin coordinates to the iron(III) centre of the other.[1] The dimers hydrogen bond to their neighbours *via* the second propionic acid group, forming infinite chains through the crystal. This substance is found in two important blood-eating pathogens, namely *Plasmodia* species[2,3] (the causative agents of malaria) and *Schistosoma mansoni*[4] a causative agent of intestinal schistosomiasis. Haemozoin is also found in *Haemoproteus columbae*,[5] a protozoan bird parasite, *Echinostoma trivolvis* a helminth that infects snails[6] and in the gut of *Rhodnius prolixus*[7] the insect vector of Chagas disease or American trypanosomiasis. It is believed to be a haem detoxification product. We have previously demonstrated that at least 95% of the haem released in *Plasmodium falciparum* by digestion of host haemoglobin is incorporated into this highly insoluble biomineral-like material.[8] Important antimalarial drugs, including chloroquine and quinine, are believed to interfere with this pathway.[9–12]

Haemozoin is chemically and structurally identical to a synthetic haematin product called β-haematin.[13] This has facilitated the study of this solid and the effects of antimalarials on its formation. There are several methods for preparing β-haematin.[3,10,14,15] The simplest involves heating a freshly precipitated suspension of haematin in an aqueous organic acid such as acetic, propionic or benzoic acid.[3,10]

The mechanism of haemozoin formation is still not well understood. Initially it was proposed that an enzyme, a so-called haem polymerase, catalyses its formation.[9] However, no such enzyme has ever been isolated. Subsequent proposals were that the process is spontaneous[10] or autocatalytic.[11] Current thinking is that haemozoin formation is a form of biomineralisation and is brought about either by lipids,[16,17] histidine rich protein[18] or both,[19] which either catalyze its formation or initiate an autocatalytic process. Definitive evidence remains lacking. A detailed understanding of the chemical kinetics of β-haematin formation, even under non-physiological conditions, may aid in elucidating the fundamental mechanism of this crystal nucleation and growth process and establish factors either limiting or promoting its formation. Such knowledge is essential if we are to understand the requirements for haemozoin formation *in vivo*.

We have previously reported two studies on the kinetics of β-haematin formation in acetate solution.[20,21] These investigations are to our knowledge the only comprehensive studies of the kinetics of β-haematin formation. In the more recent study,[21] we demonstrated that conversion of haematin to β-haematin conforms to Avrami kinetics, with a rate constant that is dependent on temperature, stirring rate, acetate concentration and pH. The

Department of Chemistry, University of Cape Town, South Africa. E-mail: tegan@science.uct.ac.za; Fax: +27-21-689-7499; Tel: +27-21-650-2528

kinetics suggested a process in which amorphous haematin is rapidly precipitated and then slowly converted to β-haematin by dissolution and re-deposition of the less soluble, microcrystalline phase. This is consistent with Ostwald's rule of stages.[22] It was further suggested that acetate might act as a phase transfer catalyst.

Preliminary studies in our laboratory have suggested that benzoic acid is far more active in promoting β-haematin formation than acetate. Given that the presence of the benzene ring permits introduction of substituents, the physical properties of benzoic acids can be manipulated relatively easily. This system may then permit greater insight into the process of β-haematin formation to be obtained. We report the results of such a study here.

Experimental

Materials

Bovine haemin was purchased from Sigma-Aldrich, South Africa and used without further purification. All other materials were of analytical grade or of the highest purity commercially available. Stock solutions of benzoic acid and substituted benzoic acids were prepared by weighing out the acids, adding water and slowly adjusting the pH with a concentrated solution of NaOH until the desired pH was attained. Solutions were then made up to volume with water in a volumetric flask and the final pH checked.

Kinetics experiments

A typical kinetic experiment was conducted in the following way: 0.030 g of haemin was weighed out and placed in a glass cell, temperature regulated by a thermostated water bath at 60 °C. This was determined prior to the kinetic experiment by measuring the temperature in the reaction cell with water in it and adjusting the water bath temperature to attain the desired temperature in the cell. The haemin was then dissolved in 2.6 ml of 0.1 M NaOH, to which 0.26 ml of 1 M HCl was subsequently added to neutralize the NaOH. After 2–5 min of temperature equilibration, 7.14 ml of 0.070 M benzoic acid solution, pH 4.5, was added to start the reaction. The reaction mixture was stirred gently using a magnetic stirrer. All experiments were conducted in this way, but parameters were varied one at a time. These included temperature, concentration of benzoic acid, pH and stirring rate. In addition, experiments were conducted in which 0.003 g of β-haematin was added after neutralization of NaOH with HCl to test the effect of seeding. Studies with substituted benzoic acids were carried out in the same manner. In all cases, the mass of haematin starting material and total reaction volume was kept constant.

The kinetics were routinely monitored using a new pyridine-based assay that we have recently described and fully validated elsewhere.[23] Briefly, a 0.005 ml sample was removed from the reaction mixture at regular intervals. This was typically every 5 or 10 min, but sometimes longer in the case of very slow reactions. In cases where the experiment was conducted unstirred, the reaction mixture was briefly stirred to produce a homogeneous suspension before the sample was removed. The sample extracted from the reaction mixture at each time point was placed in an Eppendorf tube and 2.00 ml of 5% (v/v) aqueous pyridine (pH 7.5, 0.020 M HEPES) was immediately added to the sample. This has the effect of quenching the reaction and dissolving any unreacted haematin, without affecting the β-haematin present. Haematin immediately forms a low spin pyridyl complex (probably a bis-pyridyl complex) with a distinctive visible spectrum. After vigorous mixing, followed by 15 min of settling, careful transfer of the supernatant to a cuvette permitted quantitation of the remaining haematin by visible spectroscopy. Measurements were performed at 405 nm on a Varian Cary 100 UV-visible spectrophotometer.

X-Ray diffraction, IR spectroscopy and scanning electron microscopy

In one set of experiments, the mass of haemin used was 0.200 g and all volumes were scaled up proportionally. The reaction was carried out in 0.050 M benzoic acid, pH 4.5 at 60 °C. In repeat experiments the reaction was run for 0, 60, 75 or 120 min and filtered using a 0.45 μm cellulose acetate filter disc. The samples were gently ground with an agate mortar and pestle to avoid preferred orientation effects. X-Ray powder diffraction measurements were performed on the samples without further drying. These measurements were conducted on a Huber Imaging Plate Guinier Camera 670 in the 2θ range 4–30° using Cu-Kα radiation ($\lambda = 1.5418$ Å) operating at 20 mA and 40 kV, with a step resolution of 0.005°. In one case, a sample obtained after 120 min incubation was dried in a desiccator over P_4O_{10} and silica gel for 48 h. The dried material was gently ground and used for X-ray powder diffraction measurements, IR spectroscopy and scanning electron microscopy (SEM). IR was performed on KBr discs obtained by grinding 0.002 g of sample with 0.200 g of dry KBr and pressing under 10 ton in^{-2} to produce a 16 mm diameter disc. The spectrum was recorded between 2000 and 1000 cm^{-1} using a Perkin-Elmer FT-IR Spectrum 100 spectrometer. Samples for SEM were sprinkled on Al stubs coated with a mixture of graphite and glue. The samples were then coated with gold-palladium. SEM was performed by the University of Cape Town electron microscopy unit on a LEO S440 scanning electron microscope.

Analysis of kinetics data

Analysis of the kinetics data was carried out by non-linear least squares fitting, usually to the Avrami equation (eqn (1)):[24]

$$m/m_0 = \exp(-zt^n) \quad (1)$$

where m is the mass of haematin remaining, m_0 is the initial mass of haematin, z is an empirical rate constant, t is the reaction time and n is the Avrami constant. Here, the ratio m/m_0 is given by $(A_t - A_f)/(A_0 - A_f)$, where A_t is the absorbance at time t, A_f is the absorbance at the end of the reaction and A_0 is the absorbance at the beginning of the reaction. This relationship holds since the absorbance at 405 nm is brought about by coordination of pyridine to haematin only. The value of n is indicative of the geometry of crystal growth and the nature of the nucleation process and is expected to be an integer between 1 and 4. As noted in a previous study,[21] strong correlations between z and n are observed in the fitting procedure if both n and z are allowed to refine freely. This results in large errors in the fitted values of z. However, as n must take an integer value, trial fits can be performed using each possible integer value (1, 2, 3 and 4). Our procedure was therefore to find the value of n by determining which allowed value gave

the best fit to the data. This value was found to be 4 in almost all experiments. The value of n was then fixed at this integer value and z was obtained by further refinement, resulting in acceptably small errors in the rate constant.

A few experiments were carried out in 4.5 M acetate solution, rather than in solutions of benzoic acid and its derivatives. Procedures used were identical to those previously reported by us.[21]

Results

Comparison of β-haematin formation in benzoic and acetic acid media

The reaction product obtained from incubation of haematin in 0.05 M benzoic acid (pH 4.5, 60 °C, 120 min) was characterized by X-ray powder diffraction and IR spectroscopy (Fig. 1(A) and (B)). For comparison, the XRD pattern and IR spectrum of β-haematin prepared in 4.5 M acetic acid (pH 4.5, 60 °C, 60 min) are also shown and are clearly identical. The d-spacing values corresponding to the eight most prominent peaks in the XRD pattern of β-haematin obtained from haematin† in benzoic acid are given in Table 1, where they are compared to those reported for β-haematin by Bohle *et al.*[13] They are virtually identical. These results therefore definitively identify the reaction product from the benzoic acid medium as β-haematin.

† HO–Fe(III)PPIX or H_2O–Fe(III)PPIX depending on pH.

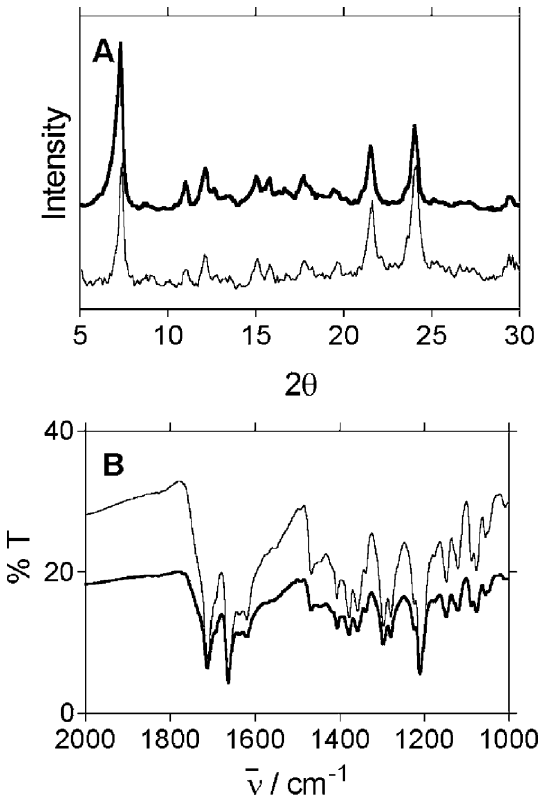


Fig. 1 XRD patterns (A) and IR spectra (B) of β-haematin prepared from a suspension of haematin in 0.050 M benzoic acid (thin line) and 4.5 M acetic acid (thick line) at pH 4.5 and 60 °C. The sample was incubated for 2 h in the benzoic acid system and 1 h in the acetic acid system. XRD patterns recorded with Cu-Kα radiation ($\lambda = 1.5418$ Å).

Table 1 Observed d-spacing for major XRD peaks of β-haematin prepared in benzoic acid compared with those reported by Bohle *et al.* for β-haematin formed by dehydrohalogenation of haemin in dry methanol[13]

d/Å	
This work	Bohle *et al.*[a]
12.1	12.0
8.03	8.02
7.30	7.32
5.60	5.63
5.00	4.99
4.59	4.55
4.13	4.08
3.60	3.65

[a] From Fig. 2 in ref. 13.

The pyridine based assay described in the Experimental section was routinely used to monitor the progress of β-haematin formation in this study. In a previous study on the kinetics of β-haematin formation in acetic acid solution,[21] we used IR spectroscopy to monitor the reaction. This is far more tedious and difficult, as it requires samples to be filtered, dried for 48 h and very carefully weighed. Uniform KBr discs then have to be prepared and the IR absorbance band corrected for background absorbance in order to be able to monitor the relative amount of β-haematin formed at given intervals during the reaction. Despite the obvious advantages of the pyridine assay, it is necessary to demonstrate that the results obtained are the same. For this reason, we followed the reaction kinetics in 4.5 M acetic acid, pH 4.5 at 60 °C using the pyridine assay and compared the data to those previously obtained under identical conditions using the IR method. The results are compared in Fig. 2. It is clear that both methods give identical results, but with less data scatter in the pyridine assay.

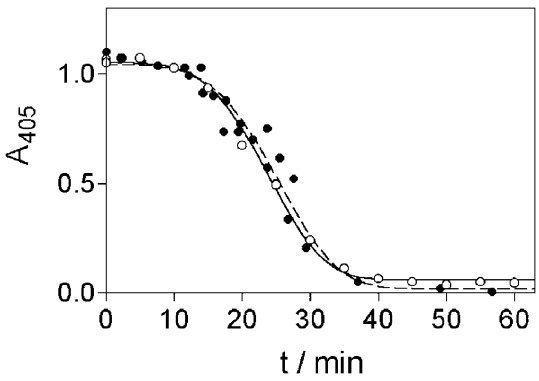


Fig. 2 Kinetics of β-haematin formation in 4.5 M acetic acid (pH 4.5, 60 °C) monitored by (○) a pyridine assay or (●) IR assay. The IR data is from previous work.[21] The original IR data were displayed as percentage β-haematin formation. These have been converted to percentage haematin remaining (100 − % β-haematin formed) and then normalized (×1.04/100) to a scale such that the starting value corresponds to the initial fitted absorbance in the pyridine assay (1.04). This normalization procedure allows direct visual comparison of the data. The best fits of the Avrami equation to the two sets of data are shown for both the pyridine assay (—) and the IR assay (---) with the Avrami constant $n = 4$.

As we have previously shown in the presence of acetic acid,[21] the kinetics of β-haematin formation conform to the Avrami equation (eqn (1)). This is a semi-theoretical equation describing a process involving nucleation and growth.[24] It contains two variables, a rate constant (z) and a so-called Avrami constant (n), which depends on the geometrical characteristics of the growth process. In the case of sporadic (random) nucleation in which the process occurs at the interface of two different phases (in this case haematin and β-haematin), n is an integer that can take on only three values. These values are 2, 3 or 4, corresponding to linear, discoid or spherical growth from the nucleation sites. An example of the analysis of kinetics performed in benzoic acid (pH 4.5, 0.050 M, 60 °C) is shown in Fig. 3(A). This demonstrates that the best fit is obtained with $n = 4$, which leads to the best correlation coefficient and to smaller and more random deviations of the data around the fitted line. By contrast, when n is fixed at 3 or 2 correlation coefficients are smaller and deviations are systematic and larger.

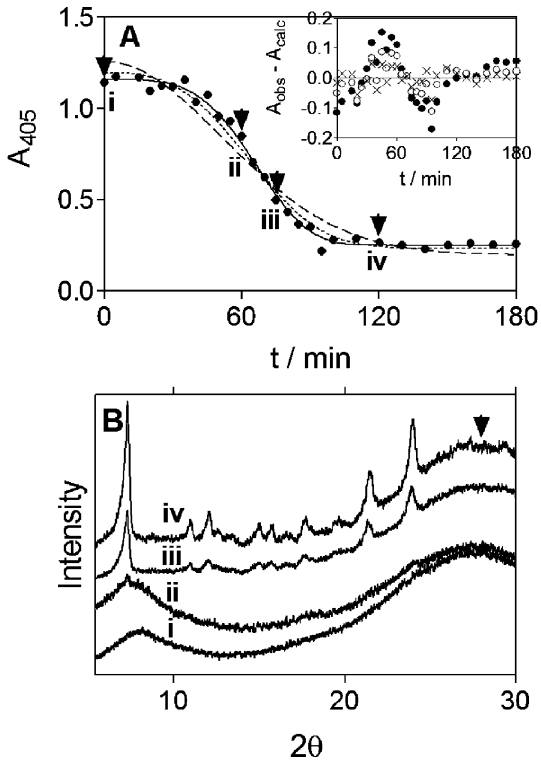


Fig. 3 Best fits of the Avrami equation to the kinetic data for β-haematin formation in 0.050 M benzoic acid, pH 4.5, 60 °C using three permitted values of the Avrami constant n for sporadic nucleation (A), *viz.* $n = 4$ (—), $n = 3$ (. . .) and $n = 2$ (---). In this case, when the data are fitted to the Avrami equation with $n = 4$ the value of r^2 is best (0.9943, *cf.* 0.9851 for $n = 3$ and 0.9541 for $n = 2$). A plot of residuals (inset) shows random deviations for data fitted to the Avrami equation with $n = 4$ (×) and larger systematic deviations with $n = 3$ (○) and $n = 2$ (●) further confirming the conclusion that $n = 4$. The arrowheads marked (i)–(iv) refer to the XRD patterns in (B) which were obtained after the corresponding reaction times (0, 60, 75 and 120 min). These show the developing diffraction pattern of β-haematin in undried samples. The broad diffraction feature marked by an arrowhead in (B) arises from liquid water in the moist sample. As we have previously pointed out, this corresponds to the radial distribution function of oxygen atoms in water (2.9 Å, giving rise to a broad feature close to $2\theta = 30°$).[21] Diffraction patterns were obtained with Cu-Kα radiation ($\lambda = 1.5418$ Å). The development of the diffraction pattern is in good qualitative agreement with the kinetic data obtained using the pyridine assay.

To further demonstrate that the observations obtained using the pyridine assay indeed correspond to formation of microcrystalline β-haematin formation, we also monitored a reaction by XRD. In this case, samples were reacted for varying lengths of time and then filtered and washed. XRD powder patterns were obtained for the filtered, but otherwise undried products. The degree of conversion to β-haematin (Fig. 3(B)) that is observed is in good qualitative agreement with the results of the pyridine assay (Fig. 3(A)).

The kinetics of β-haematin formation in 0.05 M benzoic acid are compared with those in 4.5 M acetic acid in Fig. 4. In both cases the pH (4.5), temperature (60 °C) and stirring rate are identical. It is apparent that the reaction is virtually complete in 120 min in benzoic acid, which is about double the time required to reach completion in acetic acid. However, given that the concentration of acetic acid is 90 times higher than that of benzoic acid, it would seem that benzoic acid is far more active in promoting β-haematin formation than acetic acid.

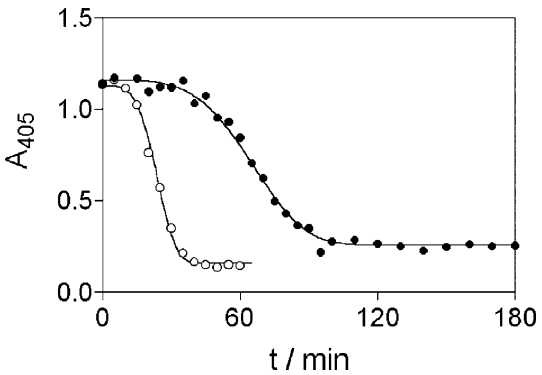


Fig. 4 Comparison of the kinetics of β-haematin formation in (●) 0.050 M benzoic acid and (○) 4.5 M acetic acid. In both cases, the pH is 4.5 and the reaction temperature is 60 °C. Solid lines are best fits of the data to the Avrami equation with $n = 4$.

Effect of stirring rate on β-haematin formation in benzoic acid

We have previously shown that stirring rate significantly affects the kinetics of β-haematin formation in the presence of 4.5 M acetic acid.[21] In the absence of stirring in acetic acid medium, the reaction requires considerably longer to reach completion and the value of the Avrami constant changes from 4 to 2. It is therefore surprising that in the presence of 0.05 M benzoic acid there is no change in the reaction kinetics when the process is carried out unstirred (Fig. 5(A)). In order to test whether this observation is an artefact of the assay method, the reaction in 4.5 M acetic acid was repeated using the pyridine assay. This confirmed our previous observations using IR spectroscopy, namely that the reaction is slower and that $n = 2$ in the unstirred acetic acid medium. Contrary to the insensitivity to lack of stirring in benzoic acid, when this medium is stirred very vigorously the reaction no longer conforms to Avrami kinetics (Fig. 5(B)), unlike the process in acetic acid medium. Instead, the reaction conforms to a modified Gompertz equation (eqn (2)).[25] The Gompertz equation is an empirical equation that has more commonly been used to describe various biological phenomena such as tumour growth.[26]

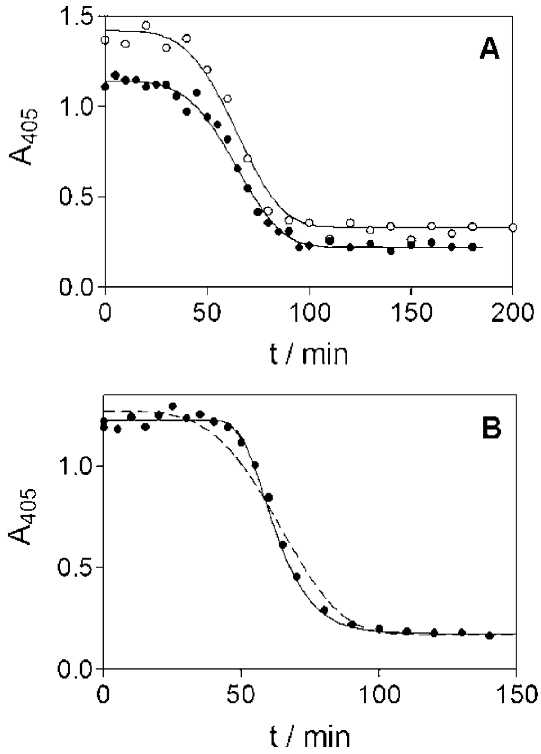


Fig. 5 Effects of stirring on β-haematin formation in 0.050 M benzoic acid. In (A) the reaction kinetics in an unstirred suspension (○) are compared with those in a suspension stirred at a moderate rate (●) corresponding to our standard reaction conditions. There is no significant change in the reaction rate constant or Avrami constant. Solid lines through the data are best fits to the Avrami equation with $n = 4$. In (B) the effect of vigorous stirring is shown. The data no longer conform to the Avrami equation, as the best fit to this equation with $n = 4$ (---) is poor. By contrast, the data fit very well to the Gompertz equation (—).

This equation mathematically models the behaviour of the system, but the fitted parameters (μ and λ) are descriptive and empirical, providing no direct insight into the microscopic mechanism.

$$A_t = A_0 - (A_0 - A_f)\exp\left[-\exp\left\{\left(\frac{\mu}{A_0 - A_f}\right)(\lambda - t) + 1\right\}\right] \quad (2)$$

The most notable difference in the kinetics between the vigorously and moderately stirred reactions is a prolonged induction phase in the former case. Shear forces arising from vigorous agitation have been reported to be able to disrupt crystal nucleation processes[27] and this is the most likely explanation for the current observation. An expected consequence of this would be a decrease in the number of nucleation sites, resulting in fewer, but larger crystals. Scanning electron microscopy (Fig. 6(A) and (B)) appears to indicate a small, but noticeable increase in crystallite size in the vigorously stirred sample. This may lend some support to the proposal that nucleus destruction is increased by vigorous stirring.

Effects of reaction conditions

Unlike acetic acid, the rate constant obtained under conditions where the reaction follows the Avrami equation has a linear dependence on benzoic acid concentration (Fig. 7(A)). As with acetic acid, the kinetics follow Arrhenius behaviour (Fig. 7(B)). The apparent activation energy obtained from the Arrhenius plot

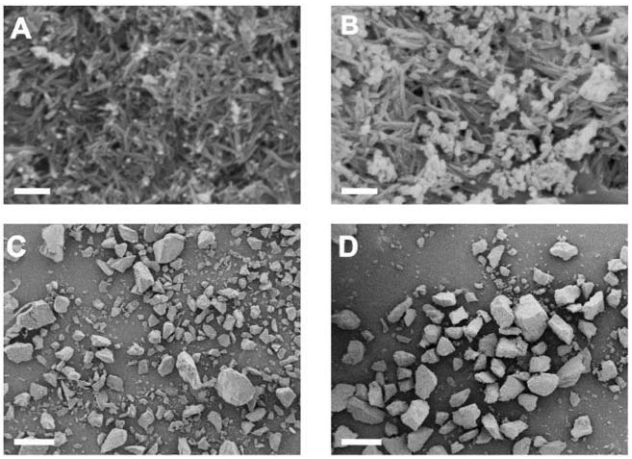


Fig. 6 Scanning electron micrographs of β-haematin produced from haematin in (A–C) 0.050 M benzoic acid and (D) 4.5 M acetic acid. The sample in (A) was produced at the normal moderate stirring rate, while that in (B) was produced with vigorous stirring. Both are shown at the same magnification (white scale bar = 1 μm), demonstrating larger crystallites in the more vigorously stirred sample, consistent with fewer nucleation sites. The images in (C) and (D) are at a low magnification (white scale bar = 0.1 mm) and demonstrate that the crystallites shown in (A) form part of large conglomerated fragments. The fragments produced in acetic acid look identical (D). There is no change in the overall appearance of these fragments during the reaction. They are seen to consist of small crystals only at high magnification.

is 334 ± 17 kJ mol^{-1}, essentially identical to that obtained in acetic acid medium (331 ± 13 kJ mol^{-1}).[21] This value appears to be unreasonably large, being comparable to, or even larger than those obtained for igneous rocks.[28,29] However, Thomas and Jennings[30] have pointed out that the activation energy obtained using rate constants obtained from the Avrami equation are only meaningful if the rate constant is expressed in a form that has units of time^{-1}. Where $n > 1$, the nth root of the rate constant should be used in the Arrhenius plot. When this approach is used, a far more reasonable activation energy of 84 ± 4 kJ mol^{-1} is obtained.

The rate constants for β-haematin formation in 0.05 M benzoic acid show a bell-shaped dependence on pH, similar to that observed in 4.5 M acetic acid medium (Fig. 7(C)).[21] We interpret this behaviour in the same way as we did for the process in acetic acid. Namely, because only one of the two propionic acid groups of the porphyrin is ionized in β-haematin, under conditions in which both groups are predominantly un-ionized (lower pH) or both are ionized (higher pH), the reaction rate is slow. The rate is maximal under conditions in which one propionic acid is predominantly ionized and the other predominantly un-ionized.

An experiment was also performed to ascertain whether seeding of the reaction with pre-formed β-haematin (10% by mass) has any effect on reaction rate. Under the conditions of the experiment, no significant effect was seen. This agrees with earlier observations in acetic acid medium.[21]

β-Haematin formation in substituted benzoic acids

Avrami kinetics were observed with all of the acids studied and in all cases the Avrami constant was found to be close to 4. Effects of temperature and concentration of 4-nitrobenzoic acid on β-haematin formation were investigated. As for benzoic acid,

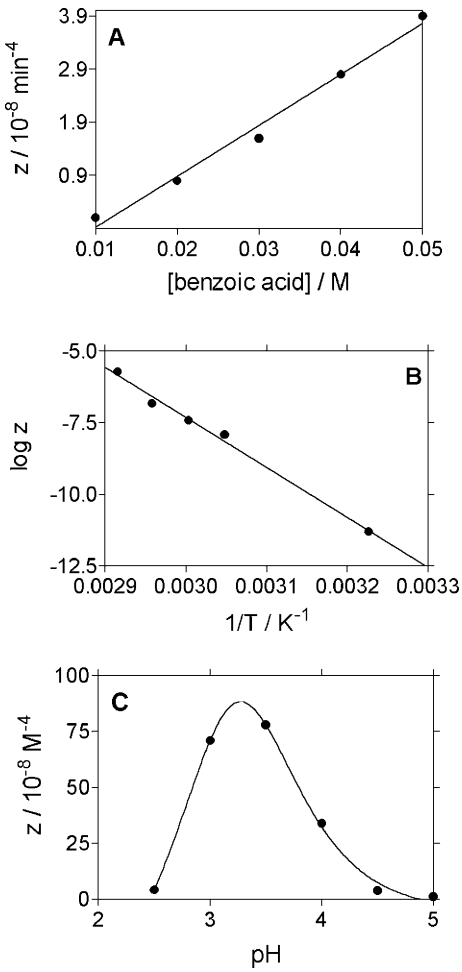


Fig. 7 Dependence of rate constant (z) for β-haematin formation on reaction conditions in benzoic acid. The dependence on benzoic acid concentration (A) was determined at pH 4.5 and 60 °C. The Arrhenius plot (B) was constructed from rate constants measured in 0.050 M benzoic acid at pH 4.5 and temperatures ranging between 37 and 70 °C. The effect of pH (C) was measured in 0.050 M benzoic acid at 60 °C. The solid line fitted through the pH data is a best fit to an equation representing two pK_a values, with only the monoprotonated species capable of forming the product. The fitted pK_a values are poorly determined because of the small number of data points, but correspond to 3.0 ± 0.8 and 3.4 ± 0.9.

Arrhenius behaviour was observed. The activation energy was found to be somewhat higher than for benzoic and acetic acid (98 ± 1 kJ mol^{-1} using $\log z^{1/4}$ in the Arrhenius plot). Despite the higher activation energy, the reaction was found to be faster in the presence of nitrobenzoic acid at all temperatures above 37 °C. A linear dependence of the rate constant on nitrobenzoic acid was observed.

Rate constants for β-haematin formation were compared in media containing one of eight different benzoic acid derivatives. The reaction was performed at 60 °C, pH 4.5 in all cases and the acids were present at 0.02 M concentration except for 4-methoxybenzoic acid. The latter acid has too low a solubility to reach this concentration at pH 4.5. Consequently, the dependence of the rate constant for β-haematin formation on the concentration of this acid was studied in the concentration range 0.005–0.007 M. An extrapolated value was used for comparison with the data for the other acids at 0.02 M. The log of the rate constants for β-haematin formation were found to have a linear dependence on the *para* substituent Hammett constant for 4-amino-, 4-methoxy-, 4-cyano- and 4-nitrobenzoic acids, benzoic acid and 4-carboxybenzaldehyde (Fig. 8). In the case of 4-methylsulfonylbenzoic acid and 4-carboxybenzenesulfonamide the rate constants lie well below the correlation line. This appears to be a steric effect, as comparison of the molar refractivities of these substituents indicates that they are much larger than the other substituents (Table 2).

Table 2 Molar refractivities (MR) of the benzoic acid substituents studied in this work

Substituent	MR[a]
H	1.03
NH_2	5.42
CN	6.3
CHO	6.9
NO_2	7.36
OCH_3	7.88
SO_2NH_2	12.28
SO_2CH_3	13.50

[a] From ref. 37.

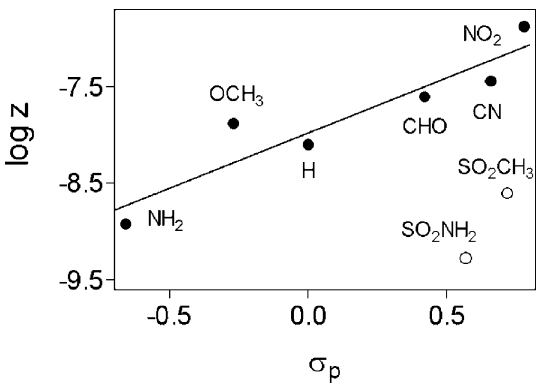


Fig. 8 Dependence of the log of the rate constant for β-haematin formation on the Hammett constants for benzoic acids with *para* substituents. There is a straight-line dependence for the compounds shown with solid circles. The two open circles represent benzoic acids with substituents substantially larger than those represented by solid circles. All of the rate constants were obtained at pH 4.5 and 60 °C with the benzoic acid derivatives present at 0.020 M concentration, except for the OCH_3 derivative which is not sufficiently soluble at this pH. For this derivative, an extrapolated rate constant was used.

Discussion

Although the kinetics of β-haematin formation in benzoic acid and substituted benzoic acid media are similar to those in acetic acid medium, there are several marked differences:

Firstly, the reaction rates in benzoic acids are apparently much faster than in acetic acid. At 60 °C and pH 4.5 the reaction is virtually complete within 100 min in the presence of 0.05 M benzoic acid and within 60 min in the presence of 0.02 M 4-nitrobenzoic acid. By contrast, under the same conditions, 4.5 M acetic acid is required for the reaction to reach completion within

60 min. At a concentration of 2.0 M acetic acid, the reaction requires about 4 h to reach completion.[21]

Secondly, absence of stirring markedly changes the rate of reaction and the geometry of crystal growth in acetic acid medium, but has no effect in benzoic acid medium. We have previously noted that in acetic acid the formation of β-haematin appears to occur *via* transformation of precipitated particles of haematin, rather than by precipitation of β-haematin from the bulk solution.[21] A similar process appears to occur in the presence of benzoic acid, as SEM shows a similar overall appearance of the precipitate at low magnification (Fig. 6(C) and (D)). The process of β-haematin formation from haematin involves loss of water from the structure. We previously speculated that stirring is required in acetic acid medium to ensure that the concentration of acetic acid is maintained in putative small channels through which water is extruded from the particles during the reaction. If this were indeed the case, then it would appear that haematin precipitates in a more open form in benzoic acid solutions, allowing rapid diffusion in and out of the precipitated particles. However, any such channels are too small to be seen by SEM, as haematin appears to be perfectly smooth to a resolution of about 40 nm.

Thirdly, vigorous stirring has no effect on β-haematin formation in acetic acid medium, but appears to destroy crystal nuclei in benzoic acid medium. This further indicates that a more open structure of haematin precipitates in benzoic acid as the nuclei are seemingly affected by shear forces, while in acetic acid they are not. An alternative explanation for these observations is that crystal growth in benzoic acid may occur from the surface of haematin particles, while in acetic acid it occurs from sites distributed throughout the material. It would be interesting to examine haematin precipitates at higher resolution, perhaps by atomic force microscopy, in order to attempt to observe whether channels do exist in haematin as well as to try to observe nucleation sites directly.

In two earlier studies, we suggested that the role of acetic acid in β-haematin formation may be to act as a phase transfer catalyst.[20,21] At first sight, it appears contradictory that carboxylic acids promote β-haematin formation because the product itself involves coordination of the haem-propionate groups of the porphyrin to the iron centre and one might expect other carboxylates to compete with this process. However, a possible explanation for this seeming contradiction is that at the pH at which β-haematin formation occurs, haematin is neutral and thus exhibits low solubility. Replacement of the neutral coordinated axial water molecule with an anionic carboxylate ligand (such as acetate) would result in a negatively charged complex that would be expected to be more soluble.‡ We thus suggested that this might facilitate dissolution and subsequent re-precipitation as β-haematin. In acetic acid medium at pH 5 there is evidence that haematin is indeed solubilised.[10] However, in the current study we could find no evidence of haematin solubilisation by 0.05 M benzoic acid. Nor could we detect any spectroscopic evidence of interaction of benzoic acid with dissolved haematin in 40% aqueous DMSO where it is monomeric.[31] Most importantly however, if coordination of the carboxylate to Fe(III) were central to the catalytic effect of these acids then the more electron donating the acid, the faster the reaction should be. Thus, the rate constant would be expected to decrease with increasing Hammett constant, the exact opposite of what is observed. In order to account for this observation, it could be argued that in addition to the haematin being in the correct protonation state for β-haematin formation, the carboxylic acid also needs to be deprotonated in order to coordinate to the Fe(III) centre. This would make the stronger acids better phase transfer catalysts as they are more highly ionized at the pH of the experiment. If this is the case, simple modelling shows that the pH maxima for the rate constants of β-haematin formation would be expected to shift to lower pH as the pK_a of the acid decreases (Fig. 9(A)). However, there is no relationship between the position of the maximum rate constant and the pK_a (Fig. 9(B)). In the light of these findings, it would appear unlikely that the acids act by forming complexes with haematin.

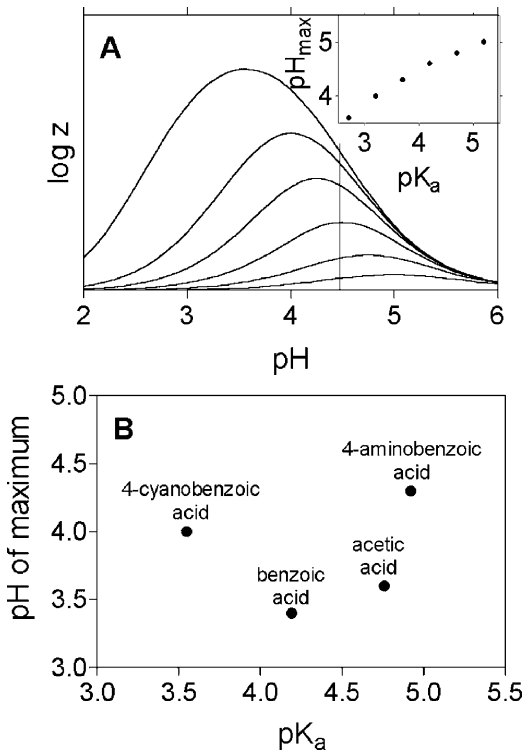
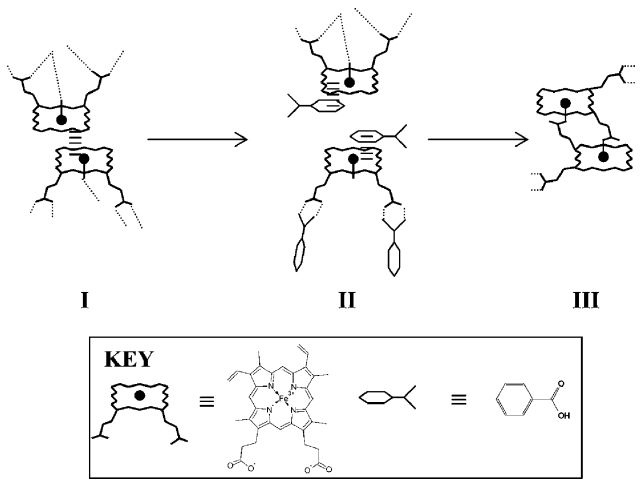


Fig. 9 The predicted dependence of rate constant on pH is shown (A) for a hypothetical system. In this model, compound I has two protonation sites (*cf*. haematin) and must be present in the mono-protonated form, while compound II (*cf*. the carboxylic acid) must be deprotonated in order to react. For I, pK_{a1} is set at 2.5 and pK_{a2} at 4.5, while for II the pK_a values are set at 5.2, 4.7, 4.2, 3.7, 3.2 and 2.7. As the pK_a of II is systematically lowered, the maximum of the bell shaped curve moves to lower pH and the maximum value of z increases. The dependence of the position of the peak maximum on pK_a is shown in the inset. Such an effect could potentially explain the observed increase in rate constant with decreasing pK_a of the acid (and hence more positive Hammett constant). This is because the rate constant at a given pH (represented by the vertical line at pH 4.5) increases as the pK_a of II decreases (modelling the effect of decreasing pK_a of the acid). However, experimentally no dependence of the pH maximum of the bell-shaped curve on pK_a is observed (B). This appears to rule out this hypothesis.

‡ In a separate study on Fe(III)PPIX dimerization in aqueous solution, we have found that the pK_a of the coordinated axial H_2O molecule is 7.1 ± 0.2 so that deprotonation of this ligand is unlikely to play any role in the kinetics described in the present study.

If carboxylic acids do not catalyze β-haematin formation by forming carboxylate complexes with the haematin Fe(III) centre, the question arises as to what their role is. A noteworthy property of carboxylic acids and carboxylates is their ability to form strong hydrogen bonds. Indeed, hydrogen bonding is known to persist even in the vapour phase.[32] This suggests their possible role in β-haematin formation (Scheme 1). Haematin is capable of forming hydrogen bonds to both of its propionate groups as well as to the axial water molecule ligated to the Fe(III) centre. We have previously shown[21] that the haematin precipitate formed at the beginning of the process is amorphous. This suggests that a complicated and random network of hydrogen bonding may exist in the solid, connecting the propionates, axial water ligands and probably solvent water molecules. In order to convert to β-haematin, these hydrogen bonds must all be disrupted. The porphyrin rings must reorient themselves, form reciprocal dimers and a chain of new, well ordered hydrogen bonds must form between one of the two propionic acids of each dimer molecule. This massive disruption of hydrogen bonding probably makes a significant contribution to the activation energy of the process. The carboxylic acid may thus have the role of aiding in the disruption of this hydrogen bonding network and facilitating the reorganization. Such an explanation can also account for the much greater activity of benzoic acid relative to acetic acid. The benzene ring in benzoic acid can, in addition to hydrogen bonding, form π–π interactions with haematin, disrupting π-stacking of the porphyrin, a further interaction that needs to be overcome in rearranging haematin to form β-haematin. Such π–π interactions are expected to be strengthened by electron withdrawing groups, as they lessen electrostatic repulsion between the π clouds. This explanation thus also accounts for the observed trend in activities of the substituted benzoic acids.



Scheme 1 A speculative representation of the process of β-haematin formation shows the possible role of benzoic acid in the reaction. Haematin (**I**) is amorphous and capable of forming numerous hydrogen bonds (---), both to other haematin molecules as well as water molecules included in the solid. It is also capable of π-stacking as indicated. Benzoic acid can disrupt both hydrogen bonding and π-stacking by itself hydrogen bonding and forming π–π interactions with haematin (**II**). This may then allow the rearrangement of haematin molecules necessary for forming β-haematin (**III**).

Finally, it is of interest to speculate about the significance of these findings for haemozoin formation *in vivo*. The most notable difference between the natural process and this and other studies on β-haematin formation is that in the former haematin is released in a continuous process owing to enzymatic digestion of haemoglobin. In the present case and other *in vitro* investigations, the process starts with a bolus of precipitated haematin. It is evident that a considerable kinetic barrier exists for conversion of solid haematin to β-haematin and it is unlikely that this barrier can be lowered sufficiently to permit rapid formation of β-haematin under physiologically reasonable conditions. In this respect, haematin precipitation can probably be considered a dead-end process *in vivo*. The formation of haemozoin probably requires direct nucleation of this thermodynamically more stable solid from solution. This suggests that one role of histidine rich protein, a protein capable of binding multiple haematin molecules[33,34] and produced in large quantities in malaria parasites, may simply be to buffer haematin concentrations in the parasite. It is also interesting to ask whether the parasite regulates haemoglobin digestion in order to ensure that the rate of haem release does not exceed the parasite's capacity to incorporate it into β-haematin. An old report does indeed suggest that haemoglobinases in the parasite food vacuole are inhibited by haematin.[35] If true, this may explain the observation of vesicles containing undigested haemoglobin in parasites treated with chloroquine.[36] Inhibition of haemozoin formation may result in just a small increase in haematin concentration, which could then feed back on haemoglobin digestion. These speculations should be subjected to experimental test. The actual formation of haemozoin is likely to occur in an environment where hydrogen-bonding to the solvent is decreased. New evidence in our laboratory suggests that this probably occurs in a lipid environment.

Acknowledgements

This material is based upon work supported in part by the National Research Foundation under Grant number 2061833. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Research Foundation. We also acknowledge the Medical Research Council of South Africa and the University of Cape Town for financial support.

References

1 S. Pagola, P. W. Stephens, D. S. Bohle, A. D. Kosar and S. K. Madsen, *Nature*, 2000, **404**, 307.
2 C. D. Fitch and P. Kanjananggulpan, *J. Biol. Chem.*, 1987, **262**, 15552.
3 A. F. G. Slater, W. J. Swiggard, B. R. Orton, W. D. Flitter, D. E. Goldberg, A. Cerami and G. B. Henderson, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 325.
4 M. Oliveira, J. C. P. d'Avila, C. R. Torres, P. L. Oliveira, A. J. Tempone, F. D. Rumjanek, C. M. S. Braga, J. R. Silva, M. Dansa-Petretski, M. A. Oliveira, W. de Souza and S. T. Ferreira, *Mol. Biochem. Parasitol.*, 2000, **111**, 217.
5 M. M. Chen, L. Shi and D. J. Sullivan, *Mol. Biochem. Parasitol.*, 2001, **113**, 1.
6 J. M. Pisciotta, E. L. Ponder, B. Fried and D. J. Sullivan, *Int. J. Parasitol.*, 2005, **35**, 1037.
7 M. F. Oliveira, J. R. Silva, M. Dansa-Petretski, W. de Souza, U. Lins, C. M. S. Braga, H. Masuda and P. L. Oliveira, *Nature*, 1999, **400**, 517.

8 T. J. Egan, J. M. Combrinck, J. Egan, G. R. Hearne, H. M. Marques, S. Ntenteni, B. T. Sewell, P. J. Smith, D. Taylor, D. A. van Schalkwyk and J. C. Walden, *Biochem. J.*, 2002, **365**, 343.
9 A. F. G. Slater and A. Cerami, *Nature*, 1992, **355**, 167.
10 T. J. Egan, D. C. Ross and P. A. Adams, *FEBS Lett.*, 1994, **352**, 54.
11 A. Dorn, R. Stoffel, H. Matile, A. Bubendorf and R. G. Ridley, *Nature*, 1995, **374**, 269.
12 D. J. Sullivan, I. Y. Gluzman, D. G. Russell and D. E. Goldberg, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 11865.
13 D. S. Bohle, R. E. Dinnebier, S. K. Madsen and P. W. Stephens, *J. Biol. Chem.*, 1997, **272**, 713.
14 D. S. Bohle and J. B. Helms, *Biochem. Biophys. Res. Commun.*, 1993, **193**, 504.
15 A. Dorn, S. R. Vippagunta, H. Matile, A. Bubendorf, J. L. Vennerstrom and R. G. Ridley, *Biochem. Pharmacol.*, 1998, **55**, 737.
16 K. Bendrat, B. J. Berger and A. Cerami, *Nature*, 1995, **378**, 138.
17 C. D. Fitch, G.-Z. Cai, Y.-F. Chen and J. D. Shoemaker, *Biochim. Biophys. Acta*, 1999, **1454**, 31.
18 D. J. Sullivan, I. Y. Gluzman and D. E. Goldberg, *Science*, 1996, **271**, 219.
19 A. V. Pandey, V. K. Babbarwal, J. N. Okoyeh, R. M. Joshi, S. K. Puri, R. L. Singh and V. S. Chauhan, *Biochem. Biophys. Res. Commun.*, 2003, **308**, 736.
20 P. A. Adams, T. J. Egan, D. C. Ross, J. Silver and P. J. Marsh, *Biochem. J.*, 1996, **318**, 25.
21 T. J. Egan, W. W. Mavuso and K. K. Ncokazi, *Biochemistry*, 2001, **40**, 204.
22 W. Kaim and B. Schwederski, in *Biomineralization: the controlled assembly of "advance materials" in biology*, Wiley, Chichester, 1996, ch. 15.
23 K. K. Ncokazi and T. J. Egan, *Anal. Biochem.*, 2005, **338**, 306.
24 A. Sharples, *Introduction to polymer crystallization*, Edward Arnold, London, 1966.
25 W. Kloek, P. Walstra and T. Van, Vliet, *J. Am. Oil Chem. Soc.*, 2000, **77**, 389.
26 R. Demicheli, G. Pratesi and R. Foroni, *Tumori*, 1991, **77**, 189.
27 J. W. Mullin and K. D. Raven, *Nature*, 1962, **195**, 4836.
28 R. K. Lowry, P. Henderson and J. Nolan, *Contrib. Mineral. Petrol.*, 1982, **80**, 254.
29 C. M. Scarfe, *Can. Mineral.*, 1977, **15**, 185.
30 J. J. Thomas and H. M. Jennings, *Chem. Mater.*, 1999, **11**, 1907.
31 G. S. Collier, J. M. Pratt, C. R. De Wet and C. F. Tshabalala, *Biochem. J.*, 1979, **179**, 281.
32 D. J. Frurip, L. A. Curtiss and M. Blander, *J. Am. Chem. Soc.*, 1980, **102**, 2610.
33 C. Y. H. Choi, J. F. Cerda, H.-A. Chu, G. T. Babcock and M. A. Marletta, *Biochemistry*, 1999, **38**, 16916.
34 A. Lynn, S. Chandra, P. Malhotra and V. S. Chauhan, *FEBS Lett.*, 1999, **459**, 267.
35 D. L. Vander, Jagt, L. A. Hunsaker and N. M. Campos, *Biochem. Pharmacol.*, 1987, **36**, 3285.
36 H. C. Hoppe, D. A. van Schalkwyk, U. I. M. Wiehart, S. A. Meredith, J. Egan and B. W. Weber, *Antimicrob. Agents Chemother.*, 2004, **48**, 2370.
37 C. Hansch and A. Leo, *Substituent constants for correlation analysis in chemistry and biology*, Wiley, New York, 1979.

