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A Comparison and Analysis of Several Ways to Promote Haematin (Haem) Polymerisation and an Assessment of Its Initiation *In Vitro*

Arnulf Dorn, Sudha Rani Vippagunta,† Hugues Matile,* André Bubendorf,* Jonathan L. Vennerstrom† and Robert G. Ridley*‡*

*Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd, CH-4070 Basel, Switzerland; and †College of Pharmacy, University of Nebraska Medical Center, Omaha, NE 68198, U.S.A.

ABSTRACT. We compared several methods for producing haematin polymerisation at physiological temperatures (i.e., 37°) and found that a trophozoite lysate-mediated reaction was inappropriate for measuring compound inhibition of haematin polymerisation. Using this method, we obtained significantly higher IC_{50} values (concentration inhibiting haematin polymerisation by 50%) for certain compounds than when other methods were used, including a food vacuole lysate-mediated reaction. This difference was probably due to the binding of these compounds to cytosolic parasite proteins, as proteinase K treatment of the trophozoite lysate reversed this effect. The initiation of haematin polymerisation was also investigated using several assays. It was found that haematin polymerisation occurred spontaneously, in the absence of preformed haemozoin, over a period of several days, but that the process was more rapid when an acetonitrile extract of malarial trophozoites was added. This extract contained no detectable protein, and its activity could be replicated using an extract from uninfected erythrocytes and by using lipids. We therefore postulate that no protein or parasite-specific material is absolutely required for the initiation of haematin polymerisation. The formation of β-haematin *de novo* using the acetonitrile extract is more pH-dependent than the generation of newly synthesised β-haematin from preformed haemozoin and cannot proceed much above pH = 6. We postulate that the initiation of haematin polymerisation is more sensitive to the equilibrium of haematin between its monomeric and μ-oxo dimer form and requires a higher concentration of monomer than for the elongation phase of polymerisation. BIOCHEM PHARMACOL **55**;6:737–747, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. malaria; haematin polymerisation; β-haematin; pH-dependence; initiation; assay methodology

Chloroquine is believed to exert its antimalarial activity by inhibiting a process of haematin (haem) polymerisation in the lysosomal digestive vacuole of the parasite [1–4]. This is probably achieved by binding to haematin [5] in the form of a μ-oxo dimer [6, 7] and preventing further haematin incorporation into the growing haemozoin polymer. The effect of this inhibition is to increase the concentration of toxic free haematin in the food vacuole which results in parasite death [5, 8]. The theory of chloroquine inhibition of haematin polymerisation and our current understanding of the mechanism of the polymerisation process have been based on several *in vitro* assays which measure the incorporation of free haematin (α-haematin) into a growing β-haematin polymer at 37° and pH 4.8, similar to the conditions of the digestive vacuole. β-Haematin is structurally equivalent to malarial haemozoin and consists of an ionic polymer in which the propionate side-chain of one haematin moiety is bonded to the ferric iron atom at the centre of an adjacent haematin moiety [9, 10].

In the first assay to be developed, the haematin polymerisation reaction was promoted using a malarial trophozoite lysate [11]. It was later found that the active agent in this lysate was haemozoin and that this alone could promote the reaction [12]. In addition to reactions promoted by preformed haemozoin, it was reported that an acetonitrile extract of trophozoite lysate could promote the reaction [13], and there was later a suggestion that the source of this activity might be lipids [14, 15]. More recently, it has been demonstrated that haematin binding histidine-rich proteins can also promote the *de novo* synthesis of β-haematin [16]. The relative significance of lipids or proteins in initiating haematin polymerisation remains open [3, 17]. Chloroquine demonstrably inhibits haematin polymerisation in all of these assays, while also inhibiting the conversion of haematin to β-haematin at the nonphysiological temperature of 70° [18] (our unpublished results).

Here, we characterise several of the haematin polymerisation reactions and assess their potential use for measuring

‡ Corresponding author: Dr. Robert G. Ridley, F. Hoffmann-La Roche Ltd, PRPI-D, Building 70/130, CH-4070 Basel, Switzerland. Tel: (+61) 688 25 75; FAX (+61) 688 2729; E-mail: robert_g.ridley@roche.com.

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drug inhibition. We also investigate the initiation of haematin polymerisation in more detail.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Plasmodium falciparum strain K1 was cultured according to the method of Trager and Jensen [19] with minor variations as described [12]. Synchronisation was attained by treatment with sorbitol [20].

Materials Used for Induction of Haematin Polymerisation

TROPHOZOITE LYSATES Trophozoites were harvested by saponin lysis, washed with PBS, pH 7.0, resuspended in PBS, frozen as beads in liquid nitrogen and stored at −80°. Trophozoite lysate were prepared by thawing the beads and mechanically disrupting them with a Dounce homogenizer. The protein concentration was ~6 mg/mL. For trophozoite lysate-initiated reactions, material containing 25 μg protein was used [12].

FOOD VACUOLE LYSATES were prepared as described previously [12, 21], and 3 μL of final suspension was used per assay.

HAEMOZOIN was prepared as previously described [22] and material containing 10–20 μmol haematin was used per assay.

β-HAEMATIN was prepared and used as previously described [12, 18].

ACETONITRILE EXTRACT FROM TROPHOZOITE LYSATE was prepared as previously described [12], except that the passage over a Resource Q column was omitted, and 10–20 μL was used per assay.

Radioisotope

Lyophilised [^{14}C]-haemin chloride (Specific activity ~105 Ci/mol; University of Leeds Innovations Industrial Services Ltd) was dissolved in 10 mM NaOH of 1 μCi/sample (200 μL). A stock solution of nonradiolabelled 2 mM haematin was prepared by dissolving haemin (Fluka Chemie AG) in 10 mM NaOH. To prepare the [^{14}C]-haemin used in the experiments, 3 mL of the non-radioactive haematin solution was added to 1 μCi [^{14}C]-haemin. The resultant [^{14}C]-haemin was stored in aliquots of 500 μL at −20°.

Haematin Polymerisation Assay

Reactions were incubated overnight at 37° on a Greiner microtiter plate in 500 mM sodium acetate, pH 4.8, with 140 μM [^{14}C]-haemin (0.16 μCi/μmol) in a final volume of 100 μL. Unincorporated haematin was removed from the insoluble haemozoin by filtrating through Millipore Multiscreen plates (0.45 μm hydrophobic low protein binding durapore membrane, MHVB N45, Millipore AG). The microtiter plates were washed with 1 mL 2% SDS in 0.1 M sodium bicarbonate buffer, pH 9.1, with 1 mL of 0.1 M sodium bicarbonate, pH 9.1, with 1 mL of 50 mM Tris-HCl, pH 7.5, and with 1 mL of distilled water. The membrane-bound material was presumed to represent polymeric β-haematin/haemozoin [11, 12, 23] and this was further confirmed by FTIR§ spectroscopy. The incorporation of haematin into the polymer was quantified by scintillation counting using the TopCount™ Microplate Scintillation System (Canberra Packard S.A.). The reactions were carried out in the presence of a variety of substances, as described in the text. For all assays, conditions were selected to ensure that counts of *ca.* 400 cpm were obtained following an overnight incubation.

FTIR Spectroscopy

The haematin polymerisation assay was carried out as described above, and free haematin was separated from haemozoin by filtration through an Eppendorf EVENT Detect test plate (positively charged nylon membrane, pore diameter 0.45 μm; supplied by Dr. Vaudaux AG, Schönenbuch, Switzerland). It was then washed as described above and dried in a vacuum oven at 60°. The insoluble material from the haematin polymerisation assay was recorded directly on the filter. FTIR spectra were measured with a Nicolet Model Magna 550 FT-IR spectrometer (Nicolet Instrument Corp.) (KBr beamsplitter and MCT detector) and an IR microscope Nic-plan equipped with an ATR (attenuated total reflection) objective made in ZnSe. Samples were examined over a range 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. Each spectrum represents an average of 500 scans to achieve a good signal-to-noise ratio and is base line corrected.

Inhibition of Haematin Polymerisation

Compounds were added to the reaction mixture as DMSO solutions up to a maximum DMSO concentration of 10%. The dpm values obtained from the assay were expressed as percent inhibition relative to haemozoin formation in a drug-free control. The values of triplicate assays were plotted semi-logarithmically (DeltaGraph Pro 3.5 and CA-Cricket Graph III 1.5.2) and the IC_{50} values [μM] calculated graphically ± SD (standard deviation).

§ *Abbreviations:* ATR, attenuated total reflection; FTIR, fourier transform infra red; HRP, histidine-rich protein(s).

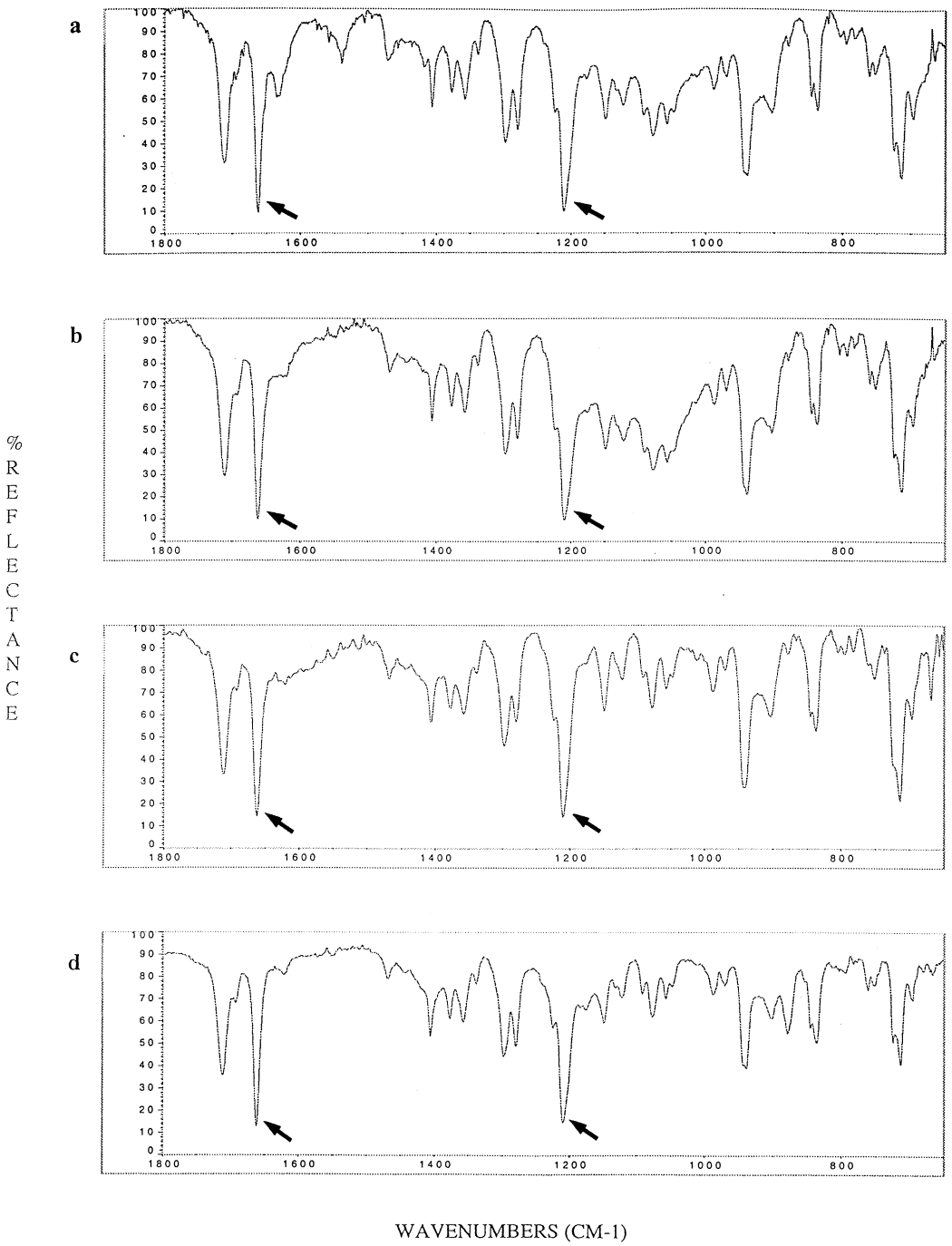


FIG. 1. Fourier transform infra red spectrum of haematin polymerisation reaction products generated from reactions using (a) trophozoite lysate, (b) haemozoin and (c) an acetonitrile extract of trophozoite lysate to induce the reaction. The spectrum of synthetic β-haematin (d) is provided for comparison. The characteristic absorption peaks at 1660 cm^{-1} and 1210 cm^{-1} are marked.

RESULTS

A Variety of Reagents Can Induce Haematin Polymerisation and β-Haematin Formation

To validate the results of the present study, we first confirmed that the products of the standard haematin polymerisation reactions used in this investigation were indeed β-haematin (haemozoin), rather than a nonspecific precipitate of haematin. This was done by FTIR spectroscopy of the filtered, washed products. Figure 1 gives the FTIR spectra of the products from assays initiated with trophozoite lysate, malarial haemozoin, and an acetonitrile extract of trophozoite lysate. Synthetic β-haematin is shown as a control. In each case the diagnostic bands at 1660 and 1210 cm^{-1} [9] unique to β-haematin/haemozoin were observed. For all reactions, incorporation of [^{14}C] haematin was linear over the time-course of the experiment (not shown).

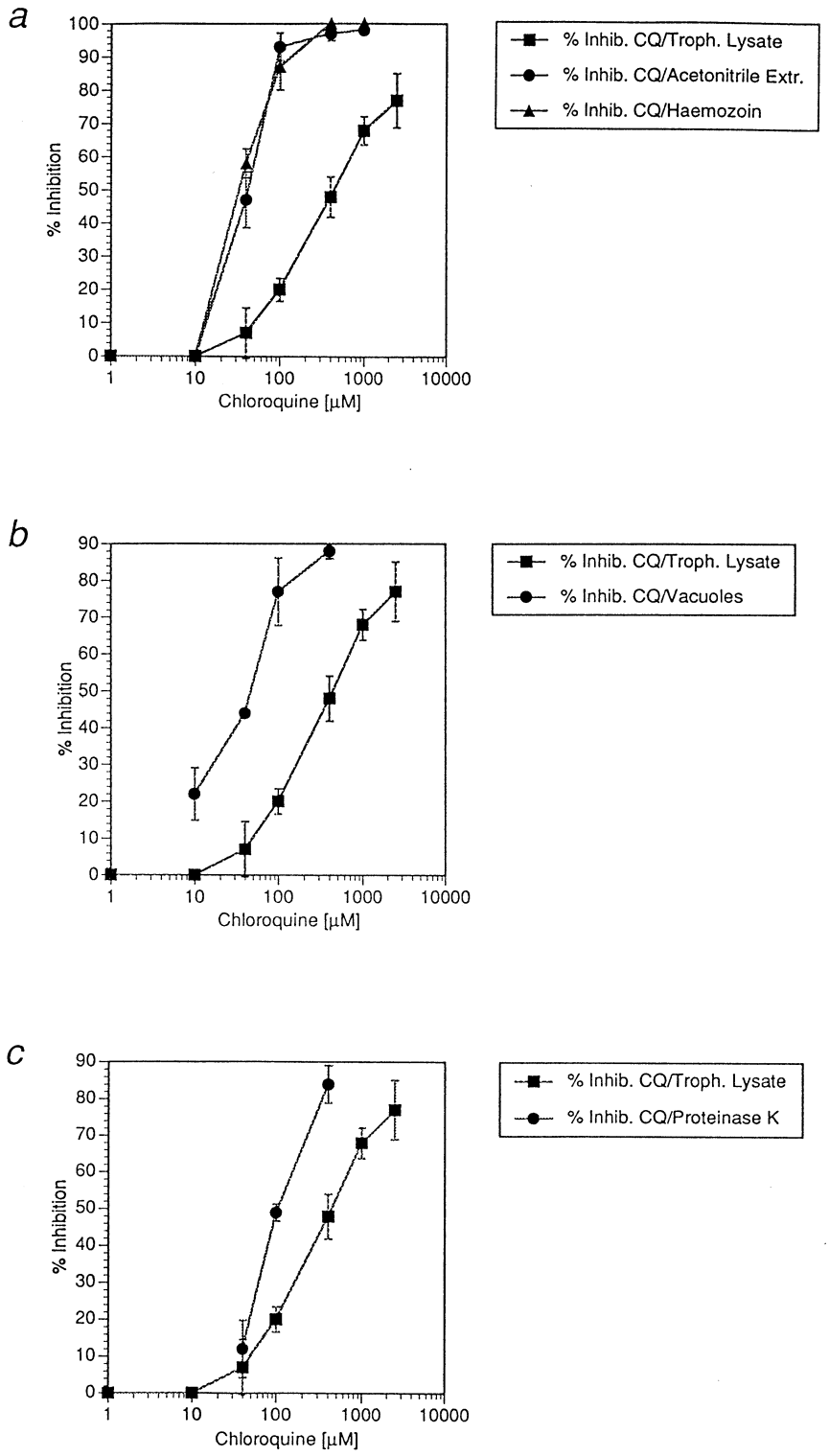


FIG. 2. Chloroquine inhibition of haematin polymerisation induced by several different methods. (a) Comparison of inhibition using trophozoite lysate, an acetonitrile extract of trophozoite lysate and haemozoin; (b) Comparison of inhibition using trophozoite lysate and food vacuole lysate; (c) Comparison of inhibition using trophozoite lysate before and after treatment with proteinase K (50 μg/mL, 10 mM Tris pH 7.8) overnight at 37°.

Chloroquine Inhibition of Haematin Polymerisation-Trophozoite Lysate is an Inappropriate Source of Activity for Assessment

We compared the ability of chloroquine to inhibit haematin polymerisation activity using the different assays described in Fig. 1. The IC_{50} titration curves of chloroquine for the various assays are shown in Fig. 2. The IC_{50} for chloroquine inhibition was significantly

higher for trophozoite lysate-initiated reactions (IC_{50} = 400 μM) than for reactions initiated using purified haemozoin, or acetonitrile extract of trophozoites (IC_{50} = 50 μM) (Fig. 2a). We wished to know which of these IC_{50} values was the most relevant value for the process in the parasite. This would best be given by the value obtained with a lysate of the food vacuole, the organelle in which haematin polymerisation occurs. A comparison of inhibition curves for the food vacuole lysate and trophozoite lysate is shown in Fig. 2b. An IC_{50} of 50 μM was obtained for food vacuole lysate, similar to those obtained using haemozoin or the acetonitrile extract, and different from that obtained using trophozoite lysate. This suggests that the trophozoite lysate is inappropriate for assessing compound inhibition of haematin polymerisation. A possible explanation for this is that chloroquine may bind to cytosolic parasite proteins in the trophozoite lysate [24], reducing the effective concentration available for the inhibition of haematin polymerisation. Pretreatment of the trophozoite lysate with proteinase K (50 μg/mL, 10 mM Tris pH 7.8) overnight at 37° resulted in a much lower chloroquine IC_{50} value, thus supporting this hypothesis (Fig. 2c).

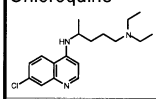
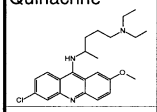
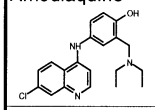
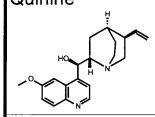
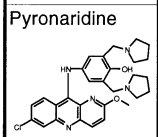
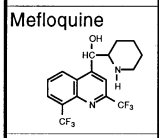
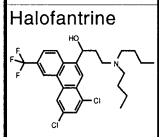
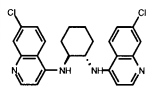
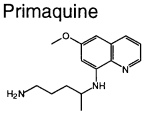
Inhibition of Haematin Polymerisation by Other Antimalarials

The ability of several antimalarial compounds to inhibit haematin polymerisation induced by either trophozoite lysate, food vacuole extract, haemozoin or an acetonitrile extract of trophozoite lysate, was tested. The results are shown in Table 1. All of the compounds used are standard antimalarial drugs with the exception of a bisquinoline, Ro 48-6910 [25–27], which has also been designated WR268,668. All of the quinoline blood schizonticides inhibited haematin polymerisation in all of the assays. Primaquine, an 8-aminoquinoline which is inactive against blood stage parasites, was not inhibitory, as discussed elsewhere [7], and served as a negative control. Significantly, the IC_{50} obtained using trophozoite lysate to promote haematin polymerisation was significantly higher than for the other assays for several compounds, reinforcing the conclusions from the assessment with chloroquine. The differences were most noticeable for chloroquine, amodiaquine, quinine and quinacrine, but less marked for mefloquine, halofantrine and pyronaridine.

Spontaneous Formation of β-Haematin at Physiological Temperature (37°)

β-Haematin formed spontaneously from haematin at 37° at pH 4.8, in the absence of any other reagents, if the monomeric haematin was allowed to incubate over several days (Fig. 3). The material was confirmed as β-haematin by filtration, further removal of monomeric haematin by washing with $NaHCO_3$ as described in the Methods section, and by FTIR.

TABLE 1. Inhibitory effect of a range of compounds on haematin polymerisation induced by several different materials at pH 4.8

	500 mM Na-acetate buffer pH 4.8			
	IC_{50} [μM]			
Compound	Trophozoite Lysate	Food vacuole extract	Haemozoin	Acetonitrile-extracted troph. lysate
Chloroquine 	400 ± 50	50 ± 4	45 ± 9	80 ± 3
Quinacrine 	2500	100 ± 1	97 ± 5	160 ± 10
Amodiaquine 	203 ± 16	60 ± 1	60 ± 1	73 ± 6
Quinine 	430 ± 45	100 ± 5	160 ± 10	78 ± 3
Pyronaridine 	60 ± 10	30 ± 2	30 ± 2	22 ± 3
Mefloquine 	200 ± 10	80 ± 10	120 ± 1	140 ± 20
Halofantrine 	60 ± 5	30 ± 2	30 ± 2	22 ± 3
Ro 48-6910 	12 ± 2	15 ± 4	10 ± 1	9 ± 1
Primaquine 	>2500	N D	>2500	>2500

Initiation of Haematin Polymerisation with a Trophozoite Acetonitrile Extract

Spontaneous haematin polymerisation in a protein-free medium, as discussed in the previous section, was promoted more rapidly with the trophozoite acetonitrile extract. Control experiments demonstrated that acetonitrile alone could not promote haematin polymerisation (not shown). The activity of the acetonitrile extracts had previously been shown to be resistant to proteinase K treatment [12] and contained no detectable protein by SDS-PAGE, UV spec-

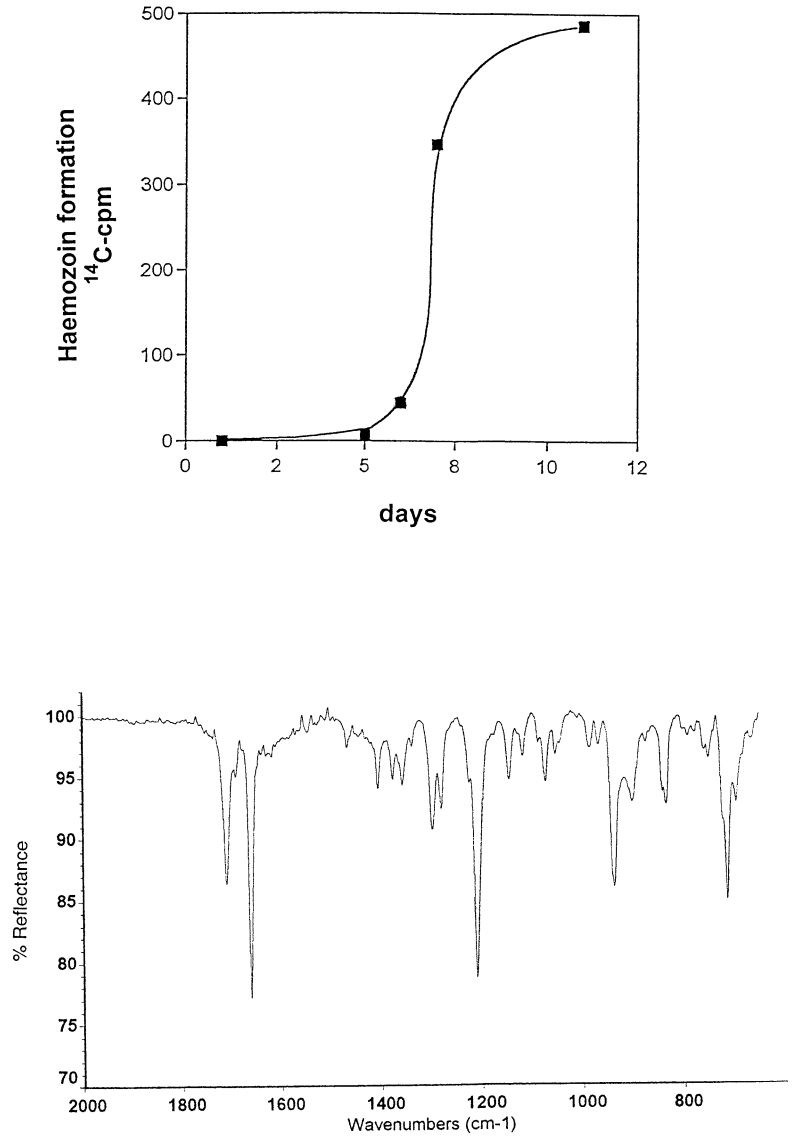


FIG. 3. Spontaneous formation of β-haematin/haemozoin in the absence of additional reagents. Haematin (140 μM) in 500 mM sodium acetate pH 4.8 was incubated at 37°. An FTIR spectrum of the product is shown. The bands at 1660 cm^{-1} and 1210 cm^{-1} demonstrate that this material is identical to β-haematin.

troscopy at 280 nm, or Bradford's assay (not shown). The extract also contained no detectable levels of haematin material. UV spectroscopy at 400 nm failed to detect porphyrin structures, and atomic absorption spectroscopy failed to detect any iron (not shown). Thus, it was improbable that low-level concentrations of monomeric or oligomeric haematin were responsible for the enhanced activity. An acetonitrile extract from an uninfected erythrocyte lysate was also found capable of supporting chloroquine-susceptible haematin polymerisation (Fig. 4), suggesting that the contents of the trophozoite acetonitrile extract responsible for initiating haematin polymerisation were not parasite-specific. This coincided with work by others suggesting that lipids may help promote haematin polymerisation [14]. We therefore tested the ability of several phospholipids plus sphingomyelin to initiate haematin polymerisation *de novo* (Fig. 5). All lipids tested (15 μg/assay) could initiate haematin polymerisation and all reactions were inhibitable by chloroquine, supporting the concept that the active agent(s) in the acetonitrile extract could be lipid(s).

Susceptibility of Haematin Polymerisation to Reducing Agents

It is postulated that β-haematin (haemozoin) consists of an ionic polymer of haematin monomers in their oxidised Fe(III) state [9]. If oxidation to the Fe(III) state is a precondition for haematin polymerisation, one would expect reducing agents to significantly inhibit the process. Table 2 demonstrates that both β-mercaptoethanol and dithiothreitol equally inhibit haematin polymerisation induced by either haemozoin or the acetonitrile extract of trophozoite lysate.

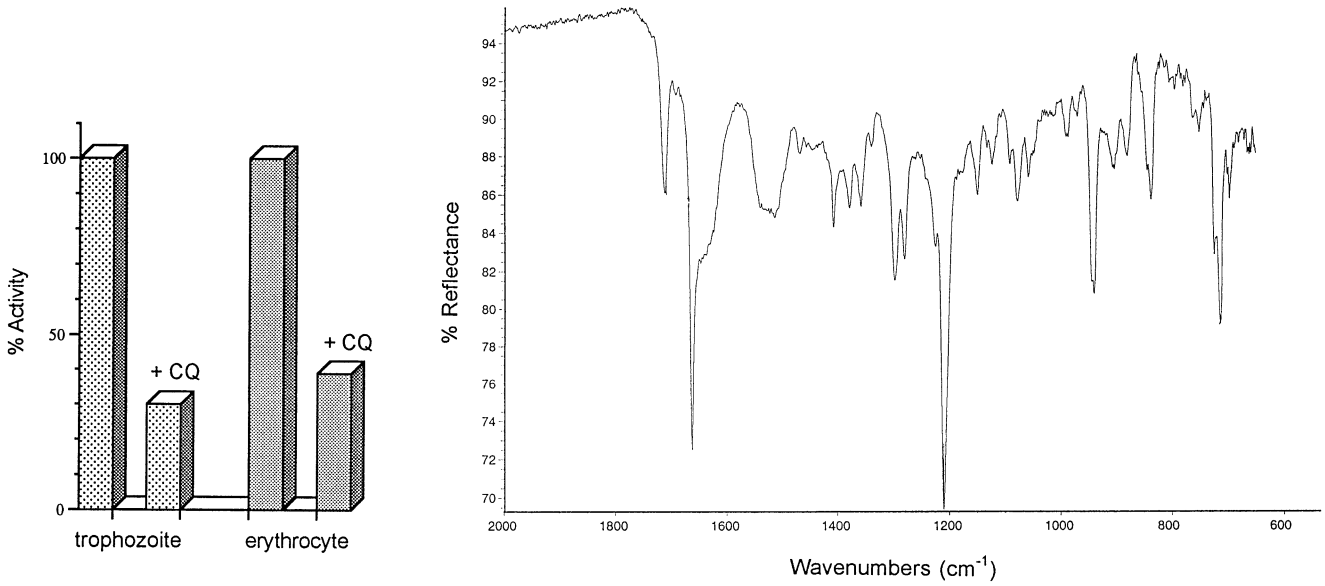


FIG. 4. Acetonitrile extracts from both trophozoite lysate and erythrocyte lysate can induce haemozoin formation, and both reactions are inhibited in the presence of 400 μM chloroquine. An FTIR spectrum of the product generated using an acetonitrile extract of erythrocytes is shown. The bands at 1660 cm^{-1} and 1210 cm^{-1} demonstrate that this material is identical to β-haematin.

pH Dependence of Haematin Polymerisation Assay

The pH profiles of haematin polymerisation reactions started either with haemozoin or with an acetonitrile extract of trophozoite lysate are given in Figure 6. In both cases, the pH optimum lies between pH 4 to 5. However, the activity was detected across a broader pH range when the reaction was initiated by adding haemozoin than when it was initiated by addition of the trophozoite acetonitrile extract; haemozoin-initiated polymerisation shows 50% activity at pH 6.5 and virtually no activity at pH 7.0, whereas acetonitrile extract-initiated polymerisation activity falls to 50% at pH 5.5 and shows virtually no activity above pH 6.0.

DISCUSSION

Trophozoite Lysate-Mediated Haematin Polymerisation Is Inappropriate for Measuring Compound IC_{50} Values

We have previously demonstrated that the quinoline antimalarials do not require interaction with protein(s) to inhibit haematin polymerisation [12] and most likely mediate their interaction through binding to haematin [5–7]. Here, we have further demonstrated that although several types of reaction can promote haematin polymerisation and the formation of β-haematin *in vitro*, care must be taken when comparing compound inhibition studies using the different reactions. In particular, IC_{50} values for compounds using trophozoite lysate-mediated reactions were higher than with the other reactions, probably due to protein binding. This would result in a lower unbound concentration of the compound in solution to bind to the haematin monomer and inhibit haematin polymerisation. Several chloroquine binding proteins [24] have been localised to the trophozoite cytosol [28] and may be responsible for this effect. It is unlikely any of these proteins plays a role in the mode of action of chloroquine or in mediating haematin polymerisation in the parasite.

Of the *in vitro* reactions used in this study, the one promoted by food vacuole lysate is probably the closest to that occurring in the parasite. However, preparation of large amounts of this material is time-consuming. We have therefore routinely used haemozoin or trophozoite acetonitrile extracts to promote haematin polymerisation reactions to measure compound inhibition. Of these two methods, haemozoin gives the most consistent results and we have relied on haemozoin-initiated reactions for detailed investigations of the mechanism of action of haematin polymerisation inhibitors such as the quinoline antimalarials [7]. IC_{50} values using acetonitrile extracts, though consistent within large batches and therefore useful for screening purposes, show some variation between batches [7].

Initiation of Haematin Polymerisation In Vitro *Can Occur in the Absence of Protein and Can Be Promoted by Lipids*

A significant difference between the haemozoin-mediated reaction and the trophozoite acetonitrile extract-mediated reaction is the lack of preformed haemozoin in the latter reaction. The former reaction presumably relies upon the extension of preformed polymeric networks of β-haematin [9, 10] by a physico-chemical, non-enzyme-mediated polymerisation process [12]. The latter reaction requires the initiation of a β-haematin network *de novo*, and its investigation provides us with some insights that may be relevant to the initiation of haematin polymerisation in the parasite.

Before discussing this reaction in more detail, however, it is significant that *de novo* formation of β-haematin from

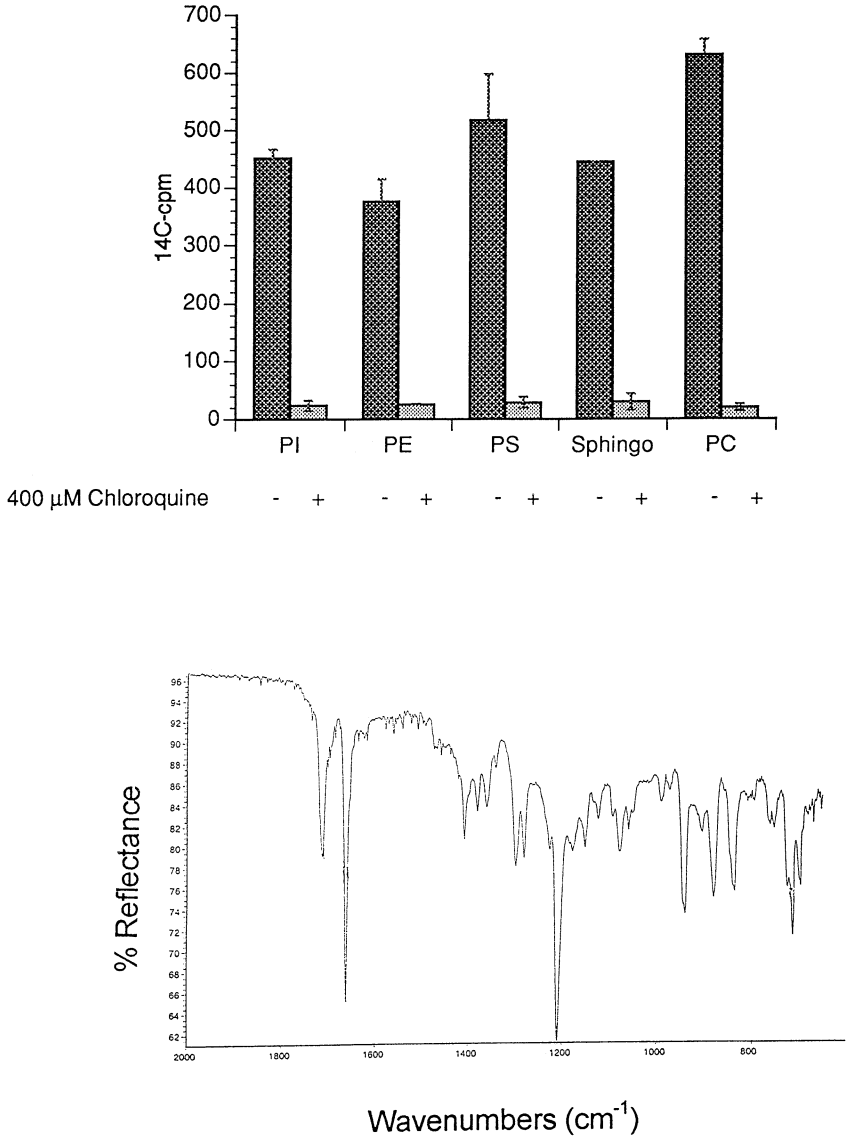


FIG. 5. Lipids (15 µg/assay) can induce haematin polymerisation, and the reaction is inhibited by 400 µM chloroquine. An FTIR spectrum of the product generated using phosphatidylcholine is shown. The bands at 1660 cm^{-1} and 1210 cm^{-1} demonstrate that this material is identical to β-haematin. (PI = L-α-phosphatidyl inositol; PE = L-α-phosphatidyl ethanol amine; PS = L-α-phosphatidyl-L-serine; Sphingo = sphingomyeline; PC = L-α-phosphatidyl choline).

monomeric α-haematin can occur without the addition of other reagents at 37° if the reaction is left for several days. Significantly, the reaction curve is sigmoidal. It would appear it takes a long time for the first few β-haematin polymeric structures to form, but that once this process has occurred, the extension of these structures is relatively rapid and self-perpetuating, consistent with the concept that haemozoin itself promotes haematin polymerisation [12]. This result is also a graphic illustration that there is no absolute requirement for protein or enzyme for the initiation of β-haematin *in vitro*. It was previously reported that spontaneous *de novo* formation of β-haematin, measured by FTIR, occurs within a few hours at 37° in the absence of any added reagents [18], but this observation was later shown to be incorrect due to a misinterpretation of bond formation between acetate in the reaction buffer and the Fe(III) ion of monomeric haematin [23]. In our experiments, taken over a much longer time period, we were clearly monitoring the formation of β-haematin and not this artefact, as the product of our reaction was filtered and washed free of both monomeric haematin and acetate.

TABLE 2. Effect of reducing agents on haematin polymerisation

Sample	Inhibition of haematin polymerisation IC_{50} Values DTT [µM]	β-mercapto [µM]
Haemozoin	70	50
$CH_3C{\equiv}N$ extracted Lysate	70	40

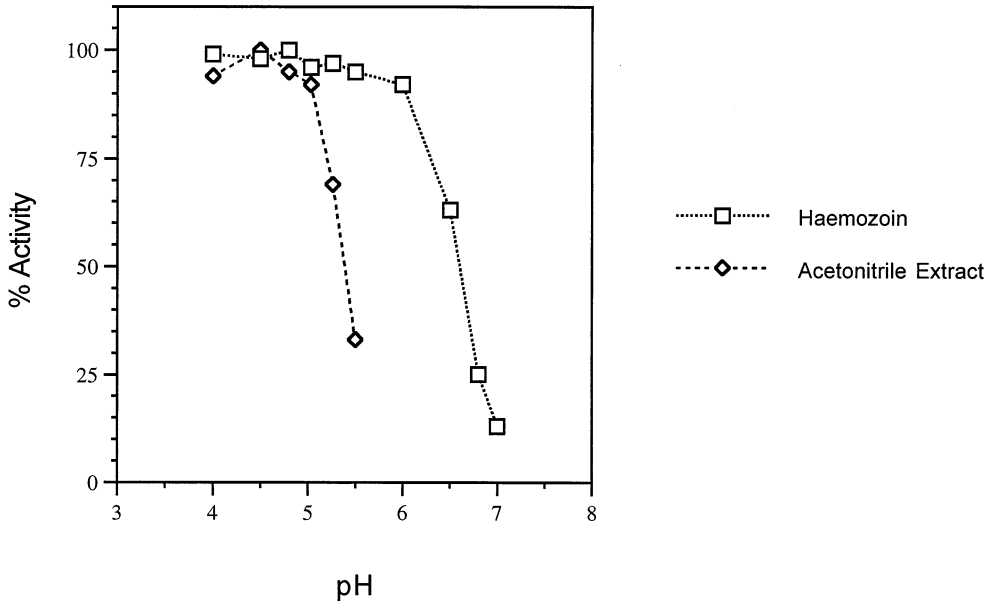


FIG. 6. The pH profile of haematin polymerisation induced by both haemozoin and an acetonitrile extract of trophozoite lysate is shown. Assays up to pH 5 were carried out using 500 mM sodium acetate buffer. Assays above pH 5 were carried out using 250 mM phosphate buffer.

Self-evidently, the initiation of haematin polymerisation needs to occur more rapidly in the food vacuole of the parasite than with haematin alone *in vitro*, and we were therefore interested that trophozoite acetonitrile extract contains material that catalyses the initiation process. We have demonstrated that lipids could be directly responsible for this activity. Consistent with this, no protein material was detected in the extract, and it was also found that acetonitrile extracts of uninfected erythrocytes could promote initiation of haematin polymerisation, suggesting the responsible agent is not even parasite-specific. Bendrat *et al.* [14] have previously suggested an involvement of lipids in haematin polymerisation through an analysis of the trophozoite acetonitrile extract in a less direct experiment than that reported here. They prepared β-haematin at nonphysiological temperatures of 70° in the presence of lipids and then claimed that acetonitrile extracts from the synthetic β-haematin prepared in this way more readily promoted haematin polymerisation.

Despite the *in vitro* data suggesting a role for lipids, the actual mechanism of the initiation of haematin polymerisation in the parasite remains unclear, as other evidence suggests that protein(s) may be involved. Sullivan *et al.* [16] demonstrated that certain histidine-rich protein(s) (HRP 2 and HRP 3) are capable of initiating haematin polymerisation *in vitro* and that one of these proteins (HRP 2) is localised in the food vacuole. To what extent these proteins are responsible for initiation in the parasite remains uncertain, as laboratory parasite strains lacking both HRP 2 and HRP 3 remain viable and produce haemozoin. At the moment, both protein-mediated and lipid-mediated initiation of haematin polymerisation remain valid hypotheses [3].

Haematin Polymerisation Is Susceptible to Oxidation State and pH

Both the oxidation state and pH are also important factors in determining why haemozoin/β-haematin forms so readily in the food vacuole of the parasite. The fact that the reducing agents 2-mercaptoethanol and dithiothreitol, capable of converting Fe(III) hematin to Fe(II) haem, inhibit haematin polymerisation is consistent with the model proposed for β-haematin in which haematin moieties are linked together through a bond between the Fe(III) of one haematin monomer and the propionate side-chain of the adjacent monomer [9, 29]. The pH optimum of 4–5 for haematin polymerisation reactions initiated both by preformed haemozoin and acetonitrile extracts of trophozoite lysate is consistent with the fact that haematin polymerisation occurs in the food vacuole of the parasite, where the pH is estimated at pH 4.8–5.2 [30, 31]. In other work, we have measured a pKa value of 5.0 for the propionic acid side-chains of haematin [15], which implies that a partial ionisation of the propionate groups may be important for the polymerisation process. This is consistent with Bohle's [10] model of β-haematin in which one propionate is ionised to allow formation of the Fe(III) propionate linkage of a growing polymeric strand and the other is protonated to allow hydrogen bonding between adjacent polymeric strands.

However, another important factor may be involved, namely the equilibrium of haematin between its monomeric and μ-oxo dimeric forms [6, 7]. Haematin exists predominantly in its μ-oxo dimer form, and in this form cannot incorporate into a growing haemozoin chain as the two central iron atoms are bound together by the bridging

oxygen atom. Others have demonstrated that the μ-oxo dimer form is favoured at the higher pH and a change of 1 pH unit may affect the equilibrium by a factor of 10 [32]. Bearing this in mind, the increased sensitivity of the acetonitrile extract-mediated reaction to increased pH could be explained if the initiation process is more sensitive to the concentration of monomeric haematin: i.e., a higher concentration of monomeric haematin is required for initiation of the polymerisation process and the initial formation of a β-haematin network than for its continued growth.

If the μ-oxo dimer equilibrium-dependent haematin monomer concentration is a key factor in the initiation of haematin polymerisation, one could postulate that lipids catalyse the initiation process by shifting the equilibrium to the monomeric form. This is currently under investigation in our laboratories. In this regard, it is worth noting that haematin polymerisation can be initiated *de novo* at physiological temperatures in organic solvents [29] and that the μ-oxo dimer equilibrium is shifted to the monomeric form in many organic solvents [33].

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