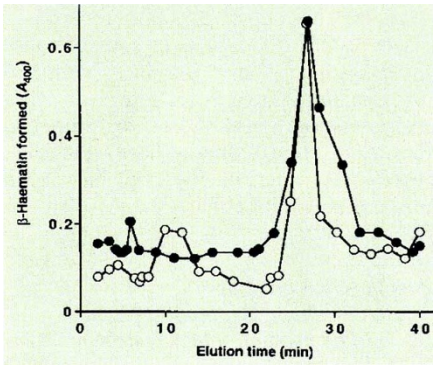
Haem polymerization in malaria

SIR — Haem that is derived from the breakdown of host cell haemoglobin by malarial parasites is sequestered by the protozoan as the polymer haemozoin. Dorn and colleagues have reported an uncharacterized 'haem-derived material', associated with both authentic haemozoin and experimentally polymerized haematin, which accelerates haem polymerization[1]. This active principle is extractable with acetonitrile, resistant to destruction by proteolysis or heat, and inhibitable by antimalarial drugs such as chloroquine. We report here that an identical activity is associated with phospholipids contaminating both haemozoin and β-haematin preparations. These lipids promote the rapid polymerization of haem, and may play a role in the formation of haemozoin by the malarial parasite.

During parasite maturation, more than 75% of host cell haemoglobin is catabolized, liberating potentially toxic haem at high rates[2]. This free haem is detoxified by parasite-specific polymerization, resulting in the inert polymer haemozoin, which forms large insoluble crystals within infected erythrocytes. The molecular mechanism of this polymerization has remained elusive, but native haemozoin formation was previously conjectured to be catalysed by a parasite-dependent activity, consistent with a hypothetical 'haem polymerase' enzyme[3].



Haem-polymerizing-activity profile of HPLC fractions from acetonitrile extracts of experimentally synthesized β-haematin (open circles) and malarial haemozoin (filled circles). Acetonitrile extracts[4] from native haemozoin (*P. falciparum*, clone HB3) and β-haematin were concentrated and further separated on a silica column by normal-phase HPLC with a hexane/isopropanol solvent system. Individual fractions were concentrated to dryness and solubilized in 500 µl (haemozoin) or 50 µl methanol (β-haematin). A 50-µl aliquot of each fraction was assayed for haem-polymerization activity by overnight incubation at 37 °C in 1 ml 0.165 M acetate pH 5, 50 µM haem, 1.1 mM methanol. The resulting haem polymer was precipitated at 12,000*g* at room temperature (20 min), washed twice with 0.1 M $NaHCO_3$ and measured photospectroscopically at 400 nm after solubilization in 1 ml 0.1 M NaOH (ref. 5).

Acetonitrile extracts of authentic haemozoin, free of detectable haem, cause rapid haem polymerization[4]. Separation of the acetonitrile-soluble material from native haemozoin by normal-phase high-pressure liquid chromatography (HPLC) with a hexane–isopropanol gradient revealed a rather broad peak that was active in the polymerization of reagent haem (see figure). Active fractions had no absorbance at 400 nm and absorbed only weakly at 210 nm, confirming the absence of porphyrins. The active fractions contained significant amounts of organically bound phosphorus and could be cleaved with phospholipase B. Treatment of material derived from acetonitrile extracts of haemozoin with water-free methanol/potassium hydroxide at 80 °C yielded several active fractions with different chromatographic properties which were identified by mass spectrometry as the methyl esters of oleic, palmitic and stearic acids.

A similarly active fraction with phospholipid-like chromatographic properties could be readily extracted (at low yield) from β-haematin chemically synthesized from commercially available haem (see figure). Commercial haem is typically prepared by extraction from intact erythrocytes, so the presence of these lipid contaminants may not be particularly surprising. In fact, much more haem-polymerizing activity was present in acetonitrile extracts of β-haematin that had been chemically synthesized in the presence of small amounts of dioleyl-phosphatidylethanolamine (ratio haem/phospholipid: 100/1).

While phospholipids may account for the acetonitrile-soluble, heat-stable and protease-resistant haem-polymerizing activity described by Dorn *et al.*[1], it is unclear whether the polymerization of haem within malaria-infected erythrocytes is effected solely by this apparent activity of endogenous lipids, or rather depends on the action of an as yet unidentified haem polymerase enzyme. These are important issues, as the sequestration of haemoglobin-derived haem may be critical to the survival and successful maturation of the malarial parasite, and may represent an important target for antimalarial drugs.

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RIDLEY *ET AL.* REPLY — Haem polymerization was first observed *in vitro* using parasite extract and was assumed to be caused by a 'haem polymerase' enzyme[3]. Later, this activity was found to be solely dependent on the presence of pre-formed haemozoin and to occur in the absence of protein[1]. There was no need to invoke an enzyme to account for haem polymerization. It was also observed that haem polymerization can be initiated, in the absence of pre-formed haemozoin, by the addition of an acetonitrile extract of *Plasmodium falciparum*[4]. At first thought to be a source of 'haem polymerase' enzyme, the activity of the extract was also shown not to be mediated by protein[1]. Bendrat and colleagues now suggest that this latter activity is in some way mediated by lipids.

Their work is a useful addition to the debate on this process, and their conclusion seems reasonable, though how the initiation of haem polymerization occurs mechanistically remains uncertain. Whatever the mechanism, we agree with Bendrat and colleagues that the activity observed in the acetonitrile extracts is not specific to parasite material. Acetonitrile extracts of uninfected erythrocytes will also initiate haem polymerization (A.D., unpublished results). The question remains, why does haemozoin, a regular crystalline polymer, form so readily in the parasite? To answer this question, it is necessary to understand the structure of haemozoin and to appreciate the role of pH in this process.

Haemozoin is believed to consist of polymeric haem linked by an Fe^{3+}–O bond between the ferric ion of one haem moiety and one of the two propionic acid side chains of an adjacent haem moiety[6,7]. These polymeric strands are further linked to each other by inter-strand hydrogen bonding between the remaining free propionic acid side chains[7]. The formation of this structure requires conditions in which 50% of the propionic acid side chains are deprotonated, which occurs at pH values equal to the pK_a of haem. We have measured this to be 4.9±0.1 (M.K., unpublished results), which coincides with the pH of the parasite food vacuole[8], the organelle within which haemozoin formation occurs. It also coincides with the pH optimum measured for the *in vitro* polymerization process initiated by acetoni-

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