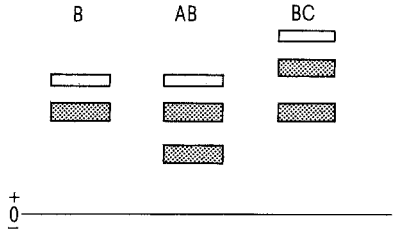
The mosquitoes used for the present study were obtained from laboratory colonies derived from Rantau Panjang, Selangor, Peninsular Malaysia[5]. Adult mosquitoes were used for horizontal starch-gel (12% hydrolyzed starch) electrophoresis employing the 'TEMM' buffer system and the enzyme visualization method of Spencer et al. with slight modification[6].

As with most culicine mosquitoes, each *Pgm* allele in *Toxorhynchites splendens* determines a two-band electrophoretic pattern (figure). In the present sample 3 codominant alleles are present. Their frequencies are $Pgm^A = 0.037$, $Pgm^B = 0.908$ and $Pgm^C = 0.055$. The distribution of the various phenotypes is summarized in the table. Only 3 phenotypes are detected in the present sample but they are in good accord with Hardy-Weinberg expectations ($\chi^2 = 1.10$). The high frequency of the Pgm^B allele also agrees with earlier reports for other mosquitoes that the most frequent allele is generally the one controlling a phenotype with an intermediate electrophoretic mobility[2]. This has been taken as supporting evidence for the idea that protein polymorphism is not primarily influenced by random genetic drift acting on a number of neutral isoalleles[7,8].

The present report helps to fill a gap in our knowledge of those mosquitoes which have not been genetically studied. Crossing experiments will be carried out to provide linkage and other data when other polymorphic gene-enzyme systems have been found in these mosquitoes.

Frequencies of *Pgm* phenotypes (= genotypes) in a laboratory colony of *Toxorhynchites splendens*

	Homozygotes			Heterozygotes		
	A	B	C	AB	AC	BC
Observed number	0	89	0	8	0	12
Expected number	0.15	89.87	0.33	7.32	0.44	10.89



Electrophoretic phenotypes of phosphoglucomutase in *Toxorhynchites splendens*.

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Binding of antimalarial drugs to hemozoin from *Plasmodium berghei*

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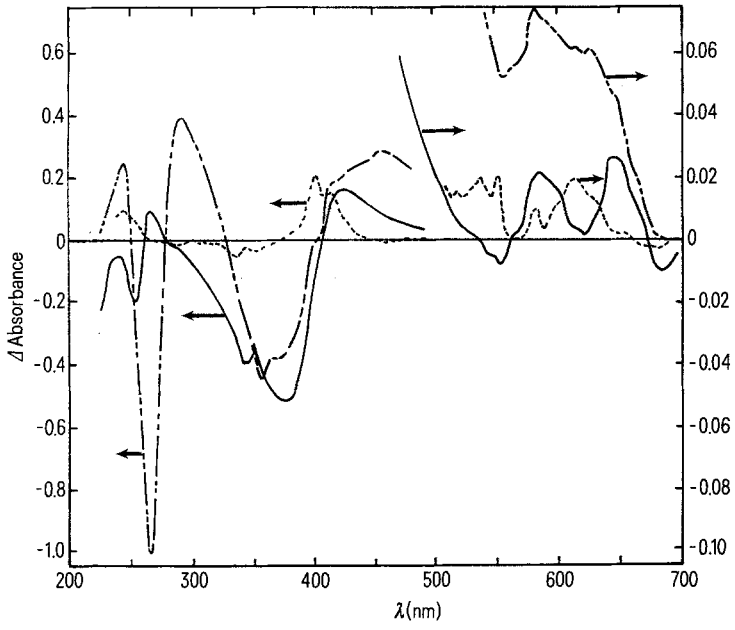
Summary. Chloroquine, quinacrine and mefloquine bind to *Plasmodium berghei* hemozoin, hemin, heme, protoporphyrin IX and protease digested methemoglobin. This binding may be the basis for drug accumulation and action in the parasite.

Studies on the molecular mechanism of action of schizontocidal antimalarial drugs are of current interest and of great relevance in the present situation of rapidly rising incidence of malarial infection and widespread drug resistance[2], e.g., to chloroquine. It is well established that chloroquine, quinacrine (mepacrine or atebrin), quinine and some similar blood-stage antimalarials bind well to DNA and can inhibit DNA, RNA and protein synthesis[3]. However, their lack of specificity for parasite DNA and the recently-observed lack of strong DNA intercalation by mefloquine, an extremely effective quinolinemethanol antimalarial, make DNA less likely as the main or only target for these drugs[3,4]. Here we report some preliminary results showing that hemozoin, the content of the parasite's 'autophagic vacuoles', is capable of binding all the above-mentioned drugs lending support to hemozoin as a more likely common site for drug uptake and concentration in the parasite than DNA.

Materials and methods. Bovine methemoglobin, hemin, trypsin and pronase were purchased from Worthington, and chloroquine, quinacrine and quinine from Sigma. Mefloquine was a gift from Dr W. Wernsdorfer of WHO. Protoporphyrin IX was from Calbiochem. Human heme (II) was prepared under nitrogen from fresh hemoglobin by the acid-acetone method[5] and also by dithionite reduction of hemin. *Plasmodium berghei* hemozoin was obtained from enriched infected mouse red cells as described by Yamada and Sherman[6] after the first-step treatment in a Parr cell disrupter bomb. A Beckman Acta V and a Perkin-Elmer (Coleman 55) spectrophotometer were used for absorbance and spectral studies; Hellma 2-chambered tandem cuvettes were used for difference spectroscopy, and a Packard scintillation counter was used for measuring ^{14}C-chloroquine (New England Nuclear) radioactivity.

Results and discussion. Our hemozoin preparation from *P. berghei* was similar to a previous preparation[6] from *P. lophurae* in terms of UV-visible spectrum and the main protein bands (mol. wts ~10,000 −20,000 and ~40,000) in the stacked gel. Upon addition of chloroquine, quinacrine and mefloquine to the hemozoin solution, spectral changes could be observed in the solely iron-porphyrin generated region from 450 nm to 700 nm clearly indicating binding. UV-visible difference spectra due to drug-hemozoin binding interaction are shown in the figure; the main longer



Spectral differences of hemozoin-drug mixture (in 1 chamber of the sample cuvette) from equal individual concentrations of hemozoin and drug (in separate chambers of the reference cuvette), all kept at pH 8.6 in Tris-buffer. Hemozoin absorbance in the reference cuvette at 382.5 nm was 1.0 and the concentration was 0.5×10^{-4} M for chloroquine (——), quinacrine (-- — --) and mefloquine (-------).

wavelength troughs are located around 375 nm, 355 nm for chloroquine and quinacrine and peaks about 425 nm, 455 nm and 400 nm for chloroquine, quinacrine and mefloquine respectively. In fact a series of curves which cut (or nearly do so) at more than 1 wavelength was obtained for each drug with varying drug: hemozoin ratios. Heme (Fe(II)) and hemin (Fe(III)) at 10^{-5}M concentrations, where Beer's Law is obeyed, exhibited in the presence of drugs a series of difference spectra similar but not identical to those of hemozoin bound to the respective drugs. Such spectroscopic changes were observed both in tris-(hydroxymethyl)aminomethane and phosphate buffers down to pH 7.0 at which the iron-porphyrins were still sufficiently soluble. However mixtures of the drugs and nitrilotriacetate-chelated Fe(II) and Fe(III) did not show significant spectral changes, indicating the importance of the porphyrin ring in inducing the large UV-spectral differences of the drugs themselves. In fact, protoporphyrin IX drug mixtures exhibited clear difference spectra. Because it is not possible to determine the concentration of hemozoin (which is not yet well characterized molecularly), binding analysis by the Job's method[7,8] (absorbance change vs continuous variations of drug:hemin molar ratio at constant drug+hemin molar concentration) was performed on ($\sim 10^{-5}$M) hemin at pH 7.5, and it was found that the stoichiometry was 1:2, 1:1.5 and 1:1.5 (drug/hemin) for chloroquine, quinacrine and mefloquine respectively. Further binding analysis will have to await more detailed investigations. It is worth mentioning here that trypsin and pronase-digested methemoglobin, unlike the undigested hemoprotein, exhibited clear difference spectra centering around 410 nm in the presence of the drugs. Quinine has also been found by difference spectroscopy (not shown) to bind hemozoin and hemin. Finally, using both unlabeled and radioactive chloroquine, it was found that precipitated hemin (50-fold excess, pH 3) brought down with it 75% of the drug. Other techniques are being resorted to for confirmation and expansion of the above findings.

The blood stage malarial schizonts digest the internalized red cell hemoglobin to produce hemozoin which is probably composed of free and polypeptide bound hemin. The binding of drugs by the hemozoin preparation, hemin, heme and protease-digested methemoglobin strongly supports hemozoin as a drug binding site and concentrating center in the parasite cytoplasm. Preliminary calculations using hemin as the model indicate that hemozoin may account for the high affinity binding site to which the above drugs show competitive binding[9-11]. Drug binding to hemozoin in vivo and the resulting accumulation, rather than providing drug resistance in schizonts as previously suggested[12], may lead to direct or indirect schizontocidal action. Free or relatively exposed iron-porphyrins in air may undergo rapid redox reactions generating toxic species of oxygen and derivatives, e.g., O_2^- and $\cdot OH$[13]. Schizontocidal drugs may affect these redox reactions and their consequences. It is worth pointing out that the resistant strains generally have less drug concentrating ability than the sensitive strains probably reflecting differences in their rate and extent of hemoglobin digestion[14]. Cross resistance to structurally similar schizontocides may be due to similarities of the binding site(s) and their drug binding mode[15].

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