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EFFECT OF MALARIA ON PHENOL CONJUGATION PATHWAYS IN PERFUSED RAT LIVER

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Abstract—The effect of malaria infection (MI) on sulphation and glucuronidation of phenol was investigated in single-pass perfused livers from rats infected with the rodent malaria parasite *Plasmodium berghei*. At a hepatic inflow (C_{in}) phenol concentration of 1 μg/mL in controls, 52% was metabolized to sulphate conjugate and 37% to glucuronide conjugate at steady state. At this C_{in}, MI had no effect on phenol clearance (CL) (control: 9.63 ± 0.38 vs MI: 9.65 ± 0.36 mL/min; $P > 0.05$) or on the formation clearance (CL_m) of the glucuronide or sulphate conjugates of phenol. When phenol C_{in} was increased 10-fold to 10 μg/mL, 6% was metabolized to sulphate conjugate and 94% to glucuronide conjugate. At this C_{in} phenol CL was decreased significantly (control: 9.44 ± 0.46 vs MI: 7.09 ± 1.51 mL/min; $P < 0.05$) and represented a decrease in intrinsic clearance (sinusoidal perfusion model) of at least 55%. This decrease was accounted for entirely by the decrease in the CL_m of the glucuronide conjugate (control: 8.88 ± 0.96 vs 5.98 ± 1.87 mL/min; $P < 0.05$), whereas the CL_m of the sulphate conjugate was unchanged. There was a negative correlation between phenol glucuronide CL_m and the severity of the erythrocytic parasitaemia ($r^2 = 0.75$, $P < 0.05$). The dose-dependent reduction in phenol glucuronidation in MI may be due to reduced availability of the cosubstrate uridine diphosphoglucuronic acid (UDPGA), because previous studies have shown that UDPGA availability depends on glycogen stores, which are known to be reduced in MI. These data suggest that sulphate conjugation is preserved in MI and that glucuronidation is preserved at low doses of substrate. At high substrate doses, glucuronidation is impaired in MI and the impairment correlates with the severity of the infection.

Malaria infection (MI§) is associated with impaired hepatic drug elimination [1–7]. Effects of MI on hepatic oxidative drug metabolizing processes have received most attention [8–10], whereas there is less known about the effects of MI on drug conjugation. There are limited data showing that MI reduces glycine conjugation and glucuronide conjugation of benzoic acid in mice [11], and the clearances of harmol and salbutamol, which are both metabolized by conjugation, are reduced in isolated perfused livers from rats with MI [7]. Recently Mansor *et al.* [12] found that MI reduced paracetamol clearance in the rat, and this was accompanied by a decrease in plasma concentrations of the glucuronide conjugate, whereas plasma concentrations of the sulphate conjugate were unchanged.

In the present study, we have investigated further the effect of MI on sulphate and glucuronidation pathways. Phenol was used as the test substrate because it is metabolized almost exclusively to sulphate and glucuronide conjugates [13, 14], and these conjugates can be assayed readily. We used the isolated perfused rat liver model, in which formation clearances of metabolites can be measured precisely, and two doses of phenol were examined.

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§ Abbreviations: MI, malaria infection; PST, phenol-sulphotransferase; UDPGA, uridine diphosphoglucuronic acid; UDPGT, uridine diphosphoglucuronosyltransferase.

MATERIALS AND METHODS

Materials. Phenol, phenol glucuronide, 4-nitrophenol and tetrabutylammonium hydrogen sulphate were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Analytical Grade triethylamine and trichloroacetic acid were obtained from Ajax Chemicals (Sydney, Australia) and HPLC-grade acetonitrile, isopropanol, hexane and methanol were obtained from Mallinckrodt (Melbourne, Australia). Sodium taurocholate was obtained from Calbiochem (San Diego, CA, U.S.A.) and bovine serum albumin from Commonwealth Serum Laboratories (Melbourne, Australia).

Animals. Male Sprague–Dawley rats were weight- and age-matched at weaning (3 weeks) and allocated randomly to two groups. One group was used as controls and the other was infected with the rodent malaria parasite *Plasmodium berghei*, as described in detail previously [2]. Briefly, a single inoculation of erythrocytes infected with *P. berghei* (3×10^7 parasitized erythrocytes in 0.2 mL) was administered via a tail vein. The ANKA strain of *P. berghei* used in these studies was originally obtained from the Liverpool School of Tropical Medicine, and after one passage in mice, the strain was preserved at −170° at the Army Malarial Research Unit, Sydney, Australia. Following infection, thick film blood slides were prepared each day from tail vein blood, and immediately prior to removal of the liver directly from venous blood, to assess the erythrocytic parasitaemia of the rats with MI. Livers were

obtained for perfusion approximately 2 weeks after infecting rats with the parasite.

Liver perfusion. The details of this procedure have been described previously [15]. Livers from both infected and control rats were surgically removed, transferred to a humidified glass chamber, connected to a 100 mL perfusion circuit and kept at 37°. The perfusate consisted of glucose (0.1% w/v), sodium sulphate (0.1 mg/mL), sodium taurocholate (30 μM), bovine serum albumin (1.0% w/v) and human red blood cells (10% v/v) in a Krebs–Ringer bicarbonate solution (pH 7.4). The perfusate was equilibrated with 100% oxygen and circulated at 10 mL/min by a peristaltic pump. Liver viability was assessed by monitoring perfusate back pressure (6.0–8.0 cm H_2O), initial bile flow (0.3–0.8 mL/hr), oxygen consumption (≥2.0 μmol/min/liver) and the macroscopic appearance of the liver.

Experimental design. After a 20 min post-surgical equilibration period, the perfusion circuit was changed to a single-pass design. Livers from 15 control rats and 15 rats with MI were perfused for 40 min with phenol at an inflow concentration of either 1 μg/mL (eight control and eight with MI), or 10 μg/mL (seven control and seven with MI). Preliminary experiments indicated that at the 1 μg/mL inflow concentration steady-state extraction of phenol was reached after 15 min of perfusion. At the inflow concentration of 10 μg/mL, however, up to 25 min were required for the production of phenol glucuronide and phenol sulphate to become constant. In all experiments, samples of hepatic inflow and outflow were therefore taken at 25, 30, 35 and 40 min and bile was collected as two 20 min pooled samples.

Drug analysis. To 1 mL of perfusate or diluted bile in a glass centrifuge tube were added 0.1 mL of internal standard solution (4-nitrophenol, 0.1 mg/mL in water), 1 mL of 1 M phosphate buffer (pH 7.0) and 10 mL hexane–isopropanol (9:1). The mixture was vortex-mixed for 60 sec, centrifuged and 9 mL of the organic layer transferred to a second glass centrifuge tube containing 0.2 mL sodium hydroxide (0.2 M). The mixture was vortex-mixed, centrifuged and the aqueous layer transferred to a microcentrifuge tube and neutralized with 20 μL hydrochloric acid (3 M). A 40 μL aliquot was injected into a liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) comprising an injector (Model U6K), a pump (Model 6000A), a UV detector operating at 273 nm (Model Lambda Max 481) and a fluorescence detector (Perkin–Elmer, Model LS-4) operating at an excitation wavelength of 266 nm and an emission wavelength of 292 nm. The fluorescence detector was used to monitor phenol and the UV detector to monitor the internal standard. The column was reverse phase phenyl with 10 μm particles (Nova-Pak, Waters) housed in a radial compression module (Model Z-module, Waters). A precolumn (μBondapack C18, 10μm, Waters) was also used. The mobile phase consisted of water–acetonitrile (74:26) containing triethylamine (0.1%), adjusted to pH 7.0 with phosphoric acid, at a flow rate of 3 mL/min. Retention times were 3.2 min for 4-nitrophenol and 4-8 min for phenol.

Phenol glucuronide was analysed using a separate procedure. To 0.25 mL of perfusate or bile in a microcentrifuge tube were added 25 μL trichloroacetic acid (4 M). The mixture was vortex-mixed, centrifuged and 20 μL were injected into the same liquid chromatograph as used for the phenol assay, with the column effluent being monitored by fluorescence only (excitation 266 nm, emission 292 nm). The column was reverse phase C18 with 10 μm particles (Nova-Pak) with a precolumn as above for phenol. The mobile phase was water–acetonitrile–methanol (78:12:10) containing tetrabutylammonium (5 mM), potassium dihydrogen phosphate (20 mM) and triethylamine (0.1%), adjusted to an apparent pH of 6.0 with orthophosphoric acid. The retention time of phenol glucuronide was 2.5 min.

Phenol sulphate was analysed by adding 1 mL hydrochloric acid (3 M) to 1 mL perfusate or diluted bile in a glass centrifuge tube. The mixture was heated on a water bath at 100° for 60 min to hydrolyse phenol conjugates, allowed to cool and analysed for phenol. The phenol concentration of this sample was taken to be the sum of the total conjugated and unconjugated phenol content of the sample, and did not increase if heating at 100° was continued for up to 6 hr. A phenol solution subjected to the same procedure showed no degradation. The unconjugated phenol and phenol glucuronide concentrations, measured separately, were subtracted from the total (conjugated plus unconjugated) phenol concentration to yield the concentration of phenol sulphate.

Pharmacokinetic and statistical analysis. Hepatic clearance (CL) of phenol was calculated at steady state as:

$$CL = Q \times \left(1 - \frac{C_o}{C_{in}}\right) \quad (1)$$

where Q is perfusate flow rate, C_{in} is phenol concentration in perfusate entering the liver and C_o is phenol concentration in hepatic outflow perfusate. Formation clearance (CL_m) of the conjugated metabolites, which represents the portion of phenol CL associated with formation of a given metabolite, was calculated as:

$$CL_m = CL \times \frac{C_{o,m}}{C_{in}} + \frac{V_{bile,m}}{C_{in}} \quad (2)$$

where $C_{o,m}$ is the metabolite concentration in hepatic outflow perfusate and $V_{bile,m}$ is the average rate of excretion of metabolite into bile. Hepatic intrinsic clearance was calculated from CL and Q by both the venous equilibrium and sinusoidal perfusion models [16]. Correlations were examined by linear regression and statistical comparisons were made using Student's t-test for unpaired data, accepting $P < 0.05$ as significant. Data in the text and tables are presented as mean ± SD.

RESULTS

Drug analysis

The within-day and day-to-day coefficients of variation were each less than 5% at concentrations of 10, 50 and 500 ng/mL for the determination of

Table 1. Details of rats and physiological indices in liver perfusion studies

	1 μg/mL Phenol		10 μg/mL Phenol	
	Control (8)*	Infected (8)	Control (7)	Infected (7)
Rat weight (g)	136.3 ± 30.0	115.5 ± 27.8†	145.4 ± 17.2	96.7 ± 29.1†
Liver weight (g)	5.22 ± 1.20	6.77 ± 1.40†	4.98 ± 1.17	6.03 ± 1.07
Bile production (mL/hr)	0.45 ± 0.07	0.39 ± 0.07	0.79 ± 0.15	0.53 ± 0.16†
Oxygen consumption (μmol/min)	18.5 ± 1.7	18.8 ± 4.4	15.7 ± 2.3	14.0 ± 2.8
Perfusion back-pressure (mmH_2O)	5.6 ± 0.8	6.4 ± 0.7†	5.1 ± 0.6	6.4 ± 2.0
Erythrocyte parasitaemia (% cells infected)	—	26.0 ± 12.8	—	36.1 ± 31.2

Values are means ± SD.
* Number of experiments.
† $P < 0.05$, significantly different compared to control.

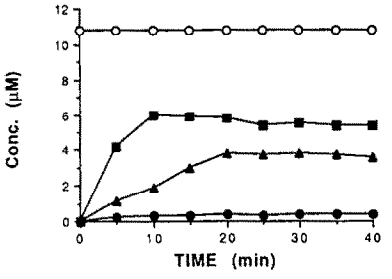

Fig. 1. Typical hepatic outflow perfusate concentrations of phenol (●), phenol sulphate (■) and phenol glucuronide (▲) during perfusion of the isolated liver with phenol C_{in} (○) of 1 μg/mL (10.6 μM).

phenol and less than 10% at concentrations of 100 and 500 ng/mL for the determination of its glucuronide and sulphate conjugates. None of the phenol concentrations encountered were less than 10 ng/mL and none of the glucuronide or sulphate conjugate concentrations were less than 100 ng/mL. The recovery of phenol was greater than 80% and that of phenol glucuronide was 100%.

Physiological indices

Physiological indices of control and infected rats are shown in Table 1. Malaria infection was associated with a significant decrease in body weight. Hepatic oxygen consumption was unchanged between the control and infected groups, but in the low dose group, liver weight was significantly greater in the infected group, and in the high dose group, bile production was significantly lower in the infected group.

Malaria and phenol elimination

Low phenol concentration (1 μg/mL). When control livers were perfused with 1 μg/mL phenol, the hepatic outflow perfusate consisted mainly of the sulphate and glucuronide conjugates and only a very small amount of unchanged phenol (Fig. 1). Phenol *CL* was very high and approached the perfusion flow rate (Table 2). Clearance to phenol sulphate and phenol glucuronide accounted for 52 and 37% of phenol *CL*, respectively (Table 2). Biliary excretion of phenol glucuronide accounted for approximately 5% of the overall phenol elimination. There were negligible amounts of unchanged phenol and phenol sulphate excreted in bile.

When livers from rats with MI were perfused with 1 μg/mL phenol, phenol *CL* was again similar to the perfusion flow rate. There were no significant differences in phenol *CL*, phenol sulphate CL_m or phenol glucuronide CL_m between the control and infected groups (Table 2).

High phenol concentration (10 μg/mL). At a 10-fold higher phenol C_{in}, hepatic outflow perfusate again consisted mainly of phenol sulphate and phenol glucuronide in the control group (Fig. 2), but phenol glucuronide CL_m accounted for a much greater proportion (94%) of phenol *CL*, whereas phenol sulphate CL_m accounted for only 6% (Table 2). There was no significant difference in phenol *CL* or biliary excretion between the high and low dose experiments in controls.

In contrast to the low dose experiments, at the 10 μg/mL C_{in}, phenol *CL* was decreased significantly from 9.44 mL/min in control to 7.09 mL/min in MI. This corresponds to a decrease in intrinsic clearance from 305 mL/min in control to 43.7 mL/min in MI, according to the venous equilibrium model, or from 31.8 mL/min in control to 14.2 mL/min in MI, according to the sinusoidal perfusion model. Thus, intrinsic clearance was decreased in MI by 55 or 86%, depending on the model. This was due to a decrease in phenol glucuronide CL_m whereas phenol sulphate CL_m was unchanged (Table 2). There was a significant negative correlation between phenol glucuronide CL_m and the severity of infection, as indicated by the erythrocytic parasitaemia ($r^2 = 0.75$, $N = 14$; $P < 0.05$; Fig. 3).

DISCUSSION

With a C_{in} of 1 μg/mL in single-pass isolated perfused livers from healthy rats, phenol was almost completely metabolized. The metabolites were made

Table 2. The effect of malaria infection on the metabolism of phenol in control and infected groups

	1 μg/mL Phenol		10 μg/mL Phenol	
	Control	Infected	Control	Infected
Phenol sulphate CL_m (mL/min)	5.02 ± 1.44	4.66 ± 1.08	0.58 ± 0.75	0.91 ± 0.56
Phenol glucuronide CL_m (mL/min)	3.57 ± 1.18	4.06 ± 0.79	8.88 ± 0.96	5.98 ± 1.87
Phenol CL (mL/min)	9.63 ± 0.38	9.65 ± 0.36	9.44 ± 0.46	7.09 ± 1.51*

Values are means ± SD.
* $P < 0.05$, significantly different compared to control.

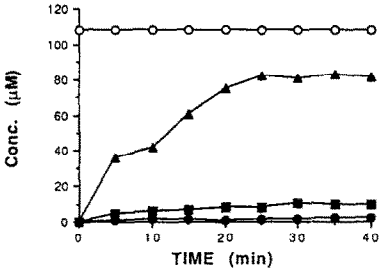

Fig. 2. Typical hepatic outflow perfusate concentrations of phenol (●), phenol sulphate (■) and phenol glucuronide (▲) during perfusion of the isolated liver with phenol C_{in} (○) of 10 μg/mL (106 μM).

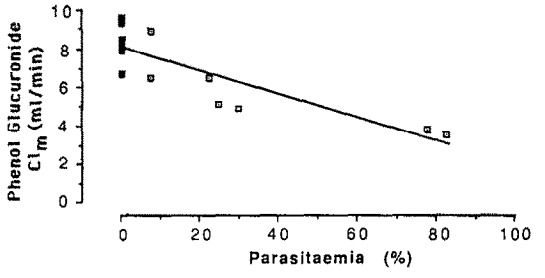

Fig. 3. Correlation between CL_m of phenol glucuronide and erythrocytic parasitaemia in the control group (■) and the infected group (□) ($r^2 = 0.75$, $P < 0.05$) following perfusion with phenol at a C_{in} of 10 μg/mL.

up of roughly equal amounts of the sulphate and glucuronide conjugates (Fig. 1) which together accounted for 89% of the administered phenol. MI was found to have no effect on phenol metabolism at this dose. Phenol CL and the formation clearances of the sulphate and glucuronide conjugates were all the same as in the controls (Table 2). This lack of an effect of MI on sulphate conjugation is consistent with the lack of an effect of MI on sulphation of paracetamol by the rat *in vivo* [12]. Phenol and paracetamol are thought to be metabolized by different isoenzymes of PST, P-PST and M-PST, respectively [17]. This indicates that there may be a general preservation of PST activity in MI. It was not possible to determine whether there was a difference in the availability of the co-substrate adenosine 3′-phosphate 5′-phosphosulphate in the two groups because inorganic sulphate was added to the perfusion fluid to ensure that in the controls the co-substrate availability would not be rate limiting. The lack of an effect of MI on glucuronidation of phenol is not consistent with previous studies of glucuronidation in MI. The CL of harmol and salbutamol, both metabolized by glucuronidation, was reduced in perfused livers from rats with MI [7], and paracetamol glucuronidation was reduced in infected rats *in vivo* [12].

Phenol was almost completely metabolized at the high dose in controls, but the metabolites were made up almost entirely of the glucuronide conjugate (Fig. 2) with only a little sulphate, which together accounted for 100% of the administered phenol. The formation rate of phenol sulphate (i.e. $CL_m \times C_{in}$ of phenol) was similar to that at the low dose (5.80 vs 5.02 μg/min, respectively), indicating that sulphation was already operating at full capacity at the low dose. The dose-dependence of the relative formation of phenol sulphate and glucuronide occurs because sulphation is a high affinity, low capacity pathway whereas glucuronidation is a low affinity, high capacity pathway [18–20]. Whereas MI had no effect on phenol CL at the low dose, at the high dose phenol CL was reduced by 25% ($P < 0.05$, Table 2). In controls, phenol CL approached the hepatic perfusion flow rate and therefore would be highly flow-dependent and relatively insensitive to changes in intrinsic clearance and protein binding in perfusate [16]. However, the perfusion flow rate and perfusate protein concentration, and therefore phenol protein binding, were held constant in these experiments. The decrease in CL, therefore, is indicative of a much greater decrease in intrinsic clearance, a measure of the capacity of the liver to metabolize the substrate [16]. According to the sinusoidal perfusion model, which provides the most conservative estimate of the decrease in intrinsic clearance, MI produced a 55% decrease in intrinsic clearance of phenol. According to the venous equilibrium model, which provides the least conservative estimate, the decrease was even greater, at 86%. Although it is not known which model is appropriate to describe the hepatic elimination of phenol, this analysis suggests that there is a substantial reduction in the capacity of the liver to

metabolize phenol in MI, in the order of 55–86%. This reduction could be accounted for entirely in terms of a reduction in glucuronidation (Table 2). The reduction in glucuronidation appeared to be related to the severity of the disease because there was a good correlation between CL_m and the degree of erythrocytic parasitaemia (Fig. 3).

Two major factors potentially determine the rate of glucuronidation, the availability of the co-substrate UDPGA, and the activity of the enzyme UDPGT. UDPGA is derived from UDP-glucose which is derived from glycogen and ATP [21]. Fasting in healthy rats decreases hepatic glycogen stores which leads to reduced UDPGA synthesis [18, 22, 23]. This leads to reduced glucuronidation of phenolic substrates, such as *p*-nitrophenol and paracetamol, the glucuronidation rates correlating with hepatic glycogen, UDP-glucose and UDPGA concentrations [18, 23]. In MI, there is reduced intestinal absorption of nutrients [24], and ATP synthesis, gluconeogenesis and glycogen production and stores are reduced [25–28]. The reduced weight of rats with MI in the present study was consistent with these changes (Table 1). The reduced glucuronidation of phenol in MI would be consistent with decreased UDPGA synthesis. Mansor *et al.* [12] suggested that the reduced glucuronidation of paracetamol in MI was unlikely to be due to reduced UDPGA synthesis because the dose they used was much lower than the dose of paracetamol known to deplete UDPGA in healthy rats. They suggested that the reduced glucuronidation of paracetamol in MI was more likely to be due to reduced UDPGT activity, due to ultrastructural damage within the hepatocyte [29]. They also suggested that there may be increased breakdown of already formed glucuronide conjugate in the liver due to increased concentrations of β-glucuronidase in the liver [30]. However, it has been shown that UDPGT becomes rate limiting in glucuronidation only when both substrate and carbohydrate reserves are high [18], and that glucuronidation rate decreases when glycogen stores are low [18, 23]. In the present study, therefore, the dose-dependent reduction of phenol glucuronidation in MI, where glycogen stores are low, indicates that reduced availability of UDPGA may be a contributing factor to impairment of glucuronidation in MI.

In conclusion, this study supports previous evidence that sulphate conjugation is preserved in MI. It shows that at low doses of substrate, glucuronidation is also preserved in MI, but at higher substrate doses glucuronidation is impaired. The degree of impairment of glucuronidation depends on the severity of the infection and may be mediated via the reduced availability of the co-substrate UDPGA.

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