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HEPATIC CYTOCHROME P-450 SYSTEM IN EXPERIMENTAL HEPATOSPLENIC SCHISTOSOMIASIS

PRESENCE OF AN ARTIFACT IN SPECTROPHOTOMETRIC ANALYSIS

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Abstract—Several recent reports suggesting that the liver microsomal cytochrome P-450 system is impaired in *Schistosoma mansoni* infections in mice prompted a detailed investigation of the hepatic cytochrome P-450 system in murine schistosomiasis. Mice were prepared with three levels of *Schistosoma mansoni* infection and studied 8, 12 and 14 weeks post-exposure. Histological sections of the liver confirmed prominent granuloma formation and portal fibrosis which was dose and time dependent. Cytochrome P-450 levels appeared reduced grossly in microsomes from homogenates of infected livers, but accurate quantitation was complicated by the presence of a prominent peak at 422 nm. Ethylmorphine *N*-demethylase activity also appeared to be reduced in all infected animals, reaching a maximum decrease at 14 weeks of 44% of control values in the most heavily infected mice. NADPH-cytochrome *c* reductase activity and cytochrome b_5 levels were similarly reduced. Utilization of techniques to separate hepatocytes from granulomatous material in infected livers eliminated the 422 nm peak and reversed several of the findings made with whole-liver derived microsomes. Cytochrome P-450 levels were reduced modestly (30%) at 8 weeks in the most heavily infected mice but returned to normal values by 14 weeks. Specific ethylmorphine *N*-demethylase activity of isolated hepatocytes was increased at 8 weeks with a return to normal by 14 weeks. Isolated granulomata were incriminated as one possible source of the 422 nm pigment in whole-liver derived microsomes but appeared unlikely to account fully for this finding. Thus, this investigation concluded that the cytochrome P-450 system is altered by experimental murine hepatosplenic schistosomiasis, but such alterations are subtle in nature and unlikely to contribute in major fashion to the observed changes in drug disposition.

Schistosomiasis is a parasitic disease, involving principally the gastrointestinal tract, the liver and the urinary bladder, which affects millions of people around the world. *Schistosoma mansoni* infection leads to egg deposition in the portal vein radicles in the liver, a granulomatous reaction to these eggs, and marked fibrosis of the portal tracts and its accompanying vessels [1–8]. The hepatic parenchyma does not appear to be importantly involved in the primary disease process.

Inevitably, therefore, traditional assessment of drug clearance in hepatic schistosomiasis placed great emphasis on altered portal hemodynamics as the major factor in reducing drug clearance by the liver [9–11]. More recently, however, the cytochrome P-450 system has been subjected to close examination. Cha and Edwards [12] and Ghazal and colleagues [13] reported that the cytochrome P-450 content and the activities of several cytochrome P-450-dependent microsomal enzymes were reduced dramatically in experimental models of schistosomiasis. These findings were so unexpected and inconsistent with most other evaluations of hepatic parenchymal function in this disorder [5, 6, 14–17] that we embarked on our own experimental study to investigate and extend these earlier findings. The experimental model of hepatosplenic schistosomiasis utilized was the mouse model first described by Olivier and Stirewalt [18]. This animal can be readily infected with *Schistosoma mansoni* cercariae, and it manifests many of the human features of the disease, including hepatomegaly, splenomegaly, and granulomatous infiltration of the portal tract with portal fibrosis [19].

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METHODS

Preparation of animals, and experimental design

C57BL/6 female mice (Jackson Laboratories, Bar Harbor, ME) were exposed at 6 weeks of age to the Puerto Rican strain of *Schistosoma mansoni* cercariae using the tail immersion technique [18]. Cercariae were shed from infected *Biomphylaria glabrata* snails following exposure of the snails to light. Mouse tails were immersed for 90 min into solutions containing 10, 30 or 100 cercariae. Cercarial viability was judged by motility, and penetration was judged by periodically counting residual head forms after exposure under the microscope. A group of mice unexposed to cercariae served as controls. Infected

and control mice were housed in separate cages permitted water and food *ad lib.* throughout the period of study. For Part I of the investigation, the mice were killed by cervical dislocation at 8, 12, and 14 weeks after cercarial exposure. Following sacrifice the abdomen was incised, and a search for ascites was conducted. The spleen was removed and weighed. The liver was perfused *in situ* with ice-cold 0.9% NaCl and then removed and weighed. Portions were set aside for histopathological examination, and the rest was used for preparation of microsomes.

In the second part of the investigation (Part II), only two groups of mice were utilized: a group infected with 100 cercariae each and a control group. These mice were caged and fed in similar fashion to mice used in the first part of the study, and they were killed in batches at 8 and 14 weeks only. At the time of sacrifice, hepatocytes and granulomata were separated from the livers using the techniques of Klaunig and co-workers [20] and Pellegrino and Brener [21] respectively.

Hepatocyte isolation

Mice were anesthetized with pentobarbital sodium (100 mg/kg), and on opening the abdomen the liver was inspected for gross pathologic features of schistosomal infection. The liver was then perfused retrogradely *in situ* with calcium- and magnesium-free Hanks' balanced salt solution (Flow Laboratories, McLean, VA) containing 0.05 M HEPES* (pH 7.3), 0.5 mM EGTA, and 1 unit/ml heparin sulfate. Livers were perfused with this solution for 4 min to reduce cellular adhesions prior to infusing a solution containing Type I collagenase (Sigma Chemical Co., St. Louis, MO), 100 units/ml dissolved in Leibovitz modified L-15 medium (Flow Laboratories) for 12 min. Both solutions were perfused at a rate of 10 ml/min and were kept at 37°. Intermittent occlusion of the portal vein accompanied by gentle massage of the liver improved the perfusion and digestion of the ground substance with collagenase especially in livers from infected animals. The liver was then transferred to a Petri dish containing the collagenase L-15 medium, disrupted by teasing and multiple pipetting, filtered sequentially through two nylon meshes having mesh sizes 253 and 64 μm respectively. Following this, cells were sedimented by centrifugation at 50 *g* for 5 min at 10°. The pellet was washed once by resuspension in L-15 medium and centrifuged at 50 *g* for a further 5 min to obtain a pellet composed of 95% pure hepatocytes. Viability of the isolated hepatocytes was checked by trypan blue exclusion stain (0.4% trypan blue in isotonic saline). More than 90% of the isolated hepatocytes excluded the trypan blue stain. Cell yield was determined by counting viable cells, using a cytometer counting slide. The yield of hepatocytes averaged 2.5×10^7 cells per animal. (Care was taken to obtain viable cells to avoid possible transformation of cytochrome P-450 to other forms such as P-420 [22, 23].)

* Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycolbis (amino-ethylether)tetra-acetate.

Isolation of granulomata

Mice were killed by cervical dislocation, and the liver was excised and immersed in ice phosphate-buffered saline (pH 7.4). The liver was then blended for 1 min at 4° in a Waring blender at maximum speed. A nylon mesh (110 μm) was used to isolate the granulomata which were then washed twice by repeated sedimentation. Microscopic examination of the isolated granulomata showed absence of liver cells. The isolated granulomata were then used to prepare a microsomal suspension.

Preparation of microsomal suspensions

Microsomes were prepared from whole livers as well as from each of the isolated components—hepatocytes and granulomata. Homogenates (5%, w/v) were prepared in iced 50 mM Tris–HCl buffer (pH 7.5) using a polytron homogenizer (Brinkmann Instruments). These were then centrifuged twice successively at 10,000 rpm for 10 min at 4° to pellet the nuclei, mitochondria and cell debris. Centrifugation of the supernatant fraction at 100,000 *g* for 1 hr at 4° provided the desired microsomal pellet.

Enzyme assays

Protein concentration of the microsomal preparations was determined by the method of Lowry *et al.* [24] as modified by Oyama and Eagle [25] using bovine serum albumin as a standard. Cytochromes P-450 and b_5 were measured by the Estabrook modification [26] of the method of Omura and Sato [27] using a Johnson Foundation split-beam spectrophotometer. Extinction coefficients for the differences between 409 and 426 nm, and between 450 and 490 nm of 185 $mM^{-1}\,cm^{-1}$ and 91 $mM^{-1}\,cm^{-1}$, respectively, were used to calculate cytochrome b_5 and cytochrome P-450 concentrations [27]. N-Demethylation of ethylmorphine was determined as described by Cochin and Axelrod [28]. The formaldehyde evolved from demethylation of ethylmorphine was measured by the colorimetric technique of Nash [29] using a Zeiss spectrophotometer at 412 nm. NADPH–cytochrome *c* reductase activity was determined in the whole liver microsomal preparations using the method described by Phillips and Langdon [30] and Masters *et al.* [31].

Histopathology

Liver samples used for histology were fixed in 10% buffered formalin. Sections were stained with hematoxylin and eosin, and by the Masson trichrome technique [32].

RESULTS

Part I: Observations using microsomes prepared from whole liver homogenates

General observations and liver histopathology. Infected mice in each of the experimental groups grew normally, and no differences in body weight were observed for any of the time periods employed. Livers became enlarged, and liver weight/body weight ratios were significantly greater in 30 and 100 cercaria-infected mice at each of the time periods. Spleen weights were greater in the more heavily

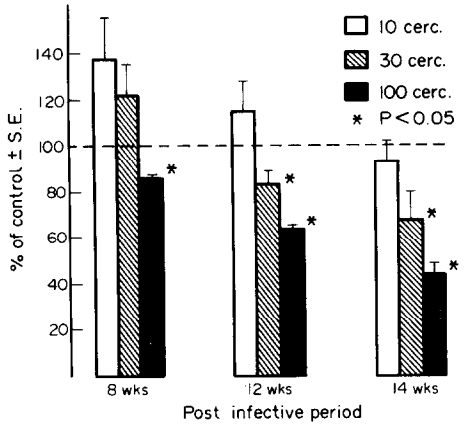

Fig. 1. Hepatic microsomal ethylmorphine N-demethylase activity in *Schistosomiasis mansoni*. Microsomes were prepared from mice exposed to 10, 30 or 100 cercariae at 8, 12, and 14 weeks post-exposure. The activity shown on the *y*-axis (mean ± SEM) is expressed as percent of control values. (Mean control activities were 8.075, 6.660 and 7.048 nmol HCHO · mg microsomal protein^{-1} · min^{-1} at 8, 12 and 14 weeks respectively.) The infectivity period is presented on the *x*-axis.

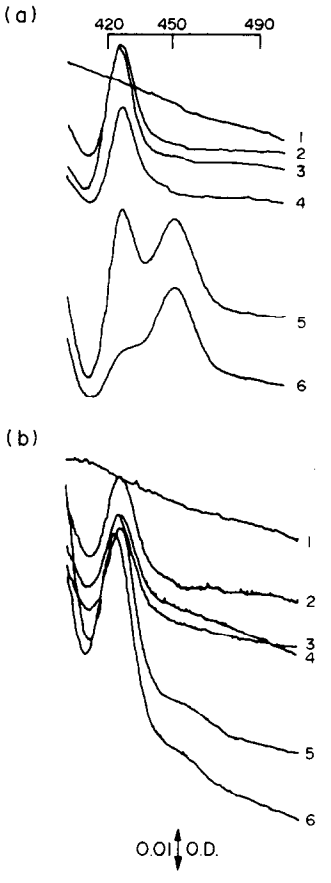

Fig. 2. Spectral properties of microsomes prepared from whole liver homogenates. (A) Difference spectra for microsomes from control mouse liver. Microsomes (3 mg protein) were diluted to a final volume of 6 ml with 100 mM sodium phosphate buffer (pH 7.4). The suspension was divided equally between reference and sample cuvettes, and the baseline was recorded (tracing #1). Twenty microliters of NADH solution (10 mg/ml) was added to the sample cuvette (tracing #2). Bubbling carbon monoxide into the sample and reference cuvettes resulted in tracings #3 and #4 respectively. A trace amount of sodium dithionite was added to the sample cuvette (tracing #5) and 20 μl of NADH solution to the reference cuvette (tracing #6). (B) Difference spectra for microsomes from 100 cercaria-infected mouse liver at 14 weeks.

infected mice, but ascites was never observed. Both the liver and spleen manifested obvious superficial granulomata in several of the heavily infected mice.

Liver histopathology demonstrated extensive granuloma formation in portal areas, and eggs were clearly recognizable at the centers of many of the granulomata. The extent of the granulomatous change was both dose and time dependent. Masson trichrome stains confirmed the presence of substantial collagen deposition in the areas of the granulomata. Also, evident in the portal/periportal regions was a dark brown pigment derived from the adult worms (which produced an obvious discoloration of the microsomal suspension in heavily infected mice).

Assays of ethylmorphine N-*demethylase activity, cytochromes* P-450 *and* b_5, *and NADPH–cytochrome* c *reductase activity*. Measurement of microsomal ethylmorphine *N*-demethylase activity revealed a significant reduction in the 30 and 100 cercaria-infected mice, particularly at 12 and 14 weeks (Fig. 1). In the latter group, a mean value of 44% of control was noted at 14 weeks. Cytochrome b_5 levels and NADPH–cytochrome *c* reductase activity were comparably reduced.

Attempts to measure cytochrome P-450 levels in microsomal preparations from the more heavily infected mice were frustrated by an unexpected spectroscopic phenomenon, as shown in Figs. 2 and 3. Addition of NADH and carbon monoxide to the sample cuvette produced a difference spectrum dominated by a peak at 420–424 nm. This peak was distinct from cytochrome b_5 since it was unaffected by addition of NADH to the reference cuvette. In the most heavily infected mice preparations, this peak dwarfed the 450 nm peak, commonly obscuring entirely that peak. For this reason, calculation of cytochrome P-450 levels in such preparations was impossible. Although the cytochrome appeared substantially reduced in the more heavily infected livers, no final conclusions regarding this were reached during this phase of the study.

Part II: Observations using microsomes prepared from isolated hepatocytes or granulomata

Spectroscopic properties of microsomes. Analysis by dual beam spectroscopy of hepatocyte microsomes from 100 cercaria-infected mice at 8 and 14 weeks showed a well defined cytochrome P-450 peak and little or no evidence of the distorting peak at 420–424 nm (Fig. 4). Microsomes prepared from the granulomata showed no cytochrome P-450 peak but did reveal a moderate sized peak at 420–424 nm.

Cytochrome b_5 *and* P-450 *levels and ethylmorphine* N-*demethylase activity in hepatocyte microsomes*. Calculation of cytochrome b_5 levels in hepatocyte

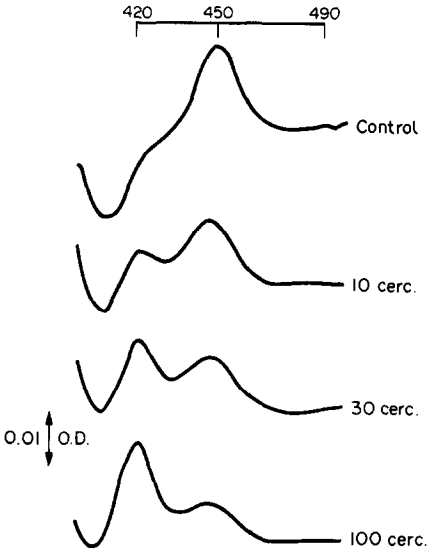

Fig. 3. Difference spectra for liver microsomes from mice infected with different doses of *Schistosoma mansoni* cercariae (0, 10, 30 or 100 Cerc) at 8 week post-exposure. The computer-derived corrected tracing (6 minus 1) for each dose shows a peak around 422 nm in addition to the cytochrome P-450 peak. Methods and tracings #1 and #6 were as described for Fig. 2.

microsomes revealed no differences between control and infected preparations at either 8 or 14 weeks (Fig. 5). Cytochrome P-450 levels were reduced modestly (30%) in infected mice at 8 weeks but had returned to normal by 14 weeks (Fig. 5).

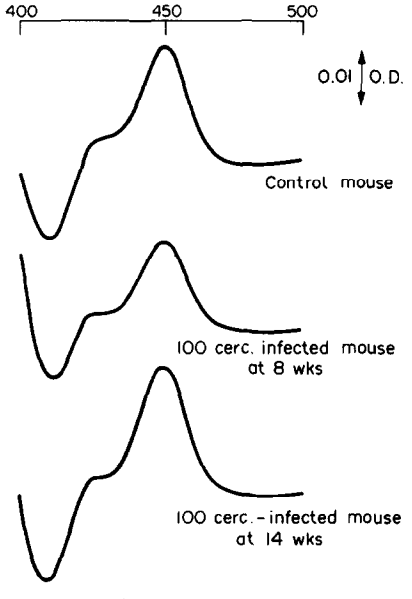

Fig. 4. Computer-derived spectra for microsomes from isolated hepatocytes (tracing #6 minus tracing #1). Control mice and mice infected with 100 *Schistosoma mansoni* cercariae were used at 8 and 14 weeks post-exposure. Methods and tracings #1 and #6 were as described for Fig. 2.

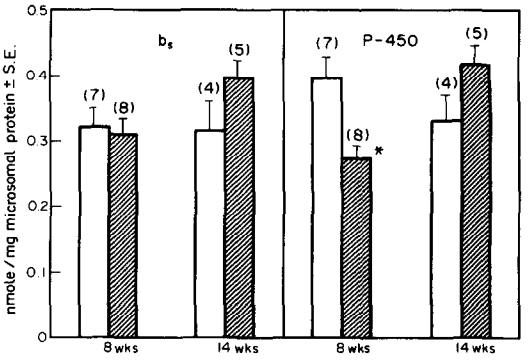

Fig. 5. Effect of *Schistosoma mansoni* infection on microsomal cytochromes in isolated hepatocytes. Cytochrome b_5 (left panel) and cytochrome P-450 (right panel) were determined at 8 and 14 weeks post 100 cercaria exposure (hatched columns) and in controls (open columns) and were expressed as mean ± SEM. The number of samples for each group is shown in parentheses. An asterisk (*) indicates a significant difference ($P < 0.05$) between infected and control preparations.

Ethylmorphine *N*-demethylase activity of hepatocyte microsomal preparations was expressed both in terms of protein content (nmol/mg protein/min) and cytochrome P-450 amount (nmol/nmol P-450/min) (Fig. 6). When calculated in the former fashion, ethylmorphine *N*-demethylase activity was normal at 8 weeks and moderately increased (45%) at 14 weeks. When calculated in terms of the cytochrome P-450 content (specific ethylmorphine *N*-demethylase activity), it was increased significantly at 8 weeks (155% of control values) but not significantly different from normal at 14 weeks.

Microsomes from granulomata had small amounts of cytochrome b_5 present (0.54 ± 0.005 nmol/mg protein), but no cytochrome P-450 or ethylmorphine *N*-demethylase activity was detected.

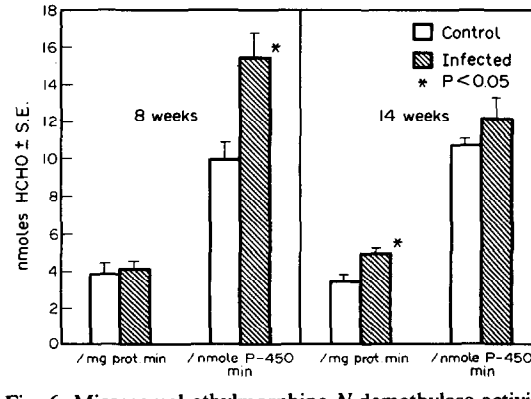

Fig. 6. Microsomal ethylmorphine *N*-demethylase activity in isolated hepatocytes. Microsomes of hepatocytes isolated from control and infected mice (100 cerc.) were prepared at 8 and 14 weeks following exposure. The activity was calculated as mean ± SEM and expressed as nmol formaldehyde formed per min either per mg microsomal protein or per cytochrome P-450 content. An asterisk (*) indicates a significant difference ($P < 0.05$) between infected and control preparations.

Mixing experiments with microsomes from hepatocytes and granulomata. Simple addition of microsomes from hepatocytes and granulomata in a ratio of 1:1 (v:v) yielded a spectroscopic tracing in which both peaks (at 450 nm and 422 nm) were visible in expected proportions. The tracing did not resemble that obtained with microsomes prepared from homogenates of infected livers (e.g. Fig. 2). However, in a preliminary experiment in which the mixture of microsomes was incubated at 37° for 1 hr, such a tracing was obtained. This observation, which is being investigated further in our laboratory, strengthens the possibility that the 420–424 nm peak seen in microsomes prepared from homogenates of infected liver actually results from degradation of cytochrome P-450 during microsome preparation.

DISCUSSION

The suggestion that the hepatic mixed-function oxidase system might be impaired in the course of hepatosplenic schistosomiasis was first made by Ghazal and co-workers [13], and later supported by Cha and Edwards [12]. Both groups reported studies in the same experimental model as the one used in our own investigation. Ghazal *et al.* [13] measured aminopyrine *N*-demethylase, hexobarbital oxidase, aniline hydroxylase and paraoxon hydrolase activities in mice 7 weeks after induction of schistosomal infection with unspecified numbers of cercariae. Enzyme activities were reduced to 35–80% of control values. Cha and co-workers [12, 33, 34] studied mice exposed to 80–100 *Schistosoma mansoni* cercariae and conducted studies at intervals beginning 5 weeks after infection. They reported a progressive reduction in NADPH–cytochrome *c* reductase activity and cytochrome P-450 content over the course of the infection, reaching a nadir of 60% of control values 8 weeks post-infection. Aminopyrine demethylase, aniline hydroxylase and benzo[*a*]pyrene hydroxylase activities were depressed even more markedly, being only 25% of control values at 8 weeks.

Cha and Bueding [33] further showed that schistosomicidal therapy leads to apparent recovery of the microsomal mixed-function oxidase system in treated mice (as had Ghazal *et al.* [13] before them), and also that the infection in athymic nude mice (which do not develop a granulomatous reaction around deposited eggs in the portal tracts) leads to a significantly smaller reduction in cytochrome P-450 content and aminopyrine demethylase and aniline hydroxylase activities [35]. Benzo[*a*]pyrene hydroxylase activity, NADPH–cytochrome *c* reductase activity, and cytochrome b_5 were not significantly different from control values. Thus, these studies demonstrated that active granuloma formation was critical for the (apparent) reduction in activity of components of the microsomal mixed-function oxidase system.

The results of the first phase of our investigation appeared to confirm these earlier studies. We considered the possibility that dilution of parenchymal tissue by the granulomatous and connective tissues present in infected livers might have contributed to the observed reductions in enzyme activities. Planimetry of stained sections from representative livers (unpublished observations) demonstrated that some of the reductions were indeed spurious and secondary to a dilution phenomenon. However, it failed to fully account for the reductions seen.

It was only during the second phase of the investigation that an explanation for the apparent enzyme reductions was established. Separation of hepatocytes from granulomatous and connective tissue using *in situ* retrograde perfusion with a collagenase-containing solution facilitated preparation of pure hepatocyte microsomes. Analysis of these microsomes for components of the mixed-function oxidase system identified far more subtle changes. Ethylmorphine *N*-demethylase activity, expressed per mg microsomal protein, was normal at 8 weeks and modestly increased at 14 weeks. When expressed in relation to cytochrome P-450 content (a more physiological calculation), ethylmorphine *N*-demethylase activity showed a significant increase (+55%) at 8 weeks with a return to normal values at 14 weeks. Total cytochrome P-450 content was reduced 30% at 8 weeks but had also returned to normal by 14 weeks. Thus, in the final analysis, this investigation led to the conclusion that induction of experimental schistosomal infection in mice leads to a perturbation of the microsomal mixed-function oxidase system early on in the course of infection (i.e. at 8 weeks) but that a return to normal takes place by week 14.

It should be recognized that the decision to proceed to phase two of the investigation in which the hepatocytes were analyzed free of granulomatous tissue was made because of the "interference" in measurement of cytochrome P-450 caused by the prominent 422 nm peak. This large peak had not been reported previously in the earlier studies by Ghazal and Cha and their co-workers [12, 13, 33–35], and we were puzzled as to its basis. Assay of microsomes from separated hepatocytes demonstrated the presence of relatively normal amounts of cytochrome P-450 free of the interfering 422 nm peak. Although analysis of microsomes from granulomatous material demonstrated a small 422 nm peak during spectral measurements, it appeared to be an unlikely source for the peak observed during quantitation of whole liver microsomes. Not only did the proportion of granulomatous tissue in whole liver microsomes appear inadequate for the large 422 nm peak observed when such microsomes were assayed, but mixing experiments involving addition of granulomatous tissue microsomes to hepatocyte microsomes in equal proportions (exceeding their true contributions to whole liver microsomes) failed to duplicate the spectral features of microsomes prepared from whole liver homogenates.

The possibility that the 422 nm peak resulted from inclusion of schistosomal pigment in the microsomes of whole liver homogenates was considered, but studies on the spectral properties of this pigment had earlier shown that it has an absorption maximum at 400 nm rather than 422 nm [36]. Furthermore, although the bulk of this pigment sedimented with the granulomatous tissue in phase 2 of the investigation, this fraction did not appear to contain suf-

ficient spectrally active material to account for the observations noted with whole liver microsomes.

Thus, it was concluded that the 422 nm peak was actually generated during preparation of the microsomes from whole liver homogenates. It also appeared likely that a relationship existed between appearance of the 422 nm peak and the decrease in cytochrome P-450 observed in preparations with a prominent 422 nm peak. In an effort to determine the feasibility of cytochrome P-450 contributing to the 422 nm peak, some preliminary studies were conducted in which hepatocyte microsomes were mixed with microsomes from granulomatous tissue and incubated at 37° for 60 min. This experiment succeeded in reproducing a spectral tracing comparable to those seen with whole liver microsomes from heavily infected livers. It appeared likely, therefore, that some component present in the granulomatous tissue caused conversion of a portion of cytochrome P-450 during microsome preparation to a species with an absorption maximum at 422 nm. Further studies are being undertaken in our laboratory to further characterize this phenomenon.

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