Biochemical Pharmacology, Vol. 46, No. 1, pp. 149–157, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CYTOCHROME P450 IN TRYPANOSOMATIDS

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(*Received* 25 *February* 1993; *accepted* 30 *March* 1993)

Abstract—Post-mitochondrial supernatant extracts prepared from bloodstream forms of *Trypanosoma brucei brucei*, *T. cruzi* epimastigotes, *Leishmania donovani* promastigotes and *Crithidia fasciculata* have been found to catalyse cytochrome P450-dependent reactions. Appreciable ethoxycoumarin deethylase and ethoxyresorufin deethylase activities were found in all of the above trypanosomatids, with *T. cruzi* epimastigotes having the highest activity (57.1 and 10.7 pmol/min/mg protein, respectively). In all four species these reactions were inhibited by the cytochrome P450 inhibitors carbon monoxide, proadifen and metyrapone. In contrast to rat liver microsomes, the trypanosomatid extracts showed no detectable pentoxyresorufin depentylase or pentamidine hydroxylase activity. Both *C. fasciculata* and *T. b. brucei* post-mitochondrial supernatants showed carbon monoxide difference spectra consistent with the presence of cytochrome P450 (9.6 and 6.3 pmol/mg protein, respectively). An additional hemoprotein which gave a carbon monoxide difference peak at 420 nm was also detected in *C. fasciculata* and *T. b. brucei* microsomes and *C. fasciculata* mitochondria. Subcellular fractionation of both early and late log *C. fasciculata* showed that the ethoxycoumarin deethylase activity was enriched in the microsomal fraction.

The cytochrome P450-dependent mixed function oxidases (P450s) are a widely distributed enzyme family responsible for the metabolism of a large number of xenobiotic and endogenous compounds [1]. While these enzymes have been discovered and characterized in a wide range of organisms, including bacteria, fungi, plants, arthropods, molluscs and vertebrates [2], there has been little research into the presence of P450s in parasites. Agosin *et al.* [3, 4] demonstrated that *Trypanosoma cruzi* epimastigotes and *T. cruzi* microsomal preparations could hydroxylate *p*-nitroanisole, aminopyrine and aniline, and that these reactions were inhibited by the P450 inhibitors carbon monoxide, proadifen and metyrapone. After partial purification of solubilized *T. cruzi* microsomes using octylamino sepharose chromatography, a typical cytochrome P450 spectrum was detectable [5]. Similar experiments have suggested the presence of P450 catalysing the dealkylation of aminopyrine and ethoxycoumarin in the malaria parasites *Plasmodium falciparum* and *P. berghei* [6, 7]. The sensitivity of *T. cruzi* and *Leishmania* spp. to ketoconazole and other azole compounds [8, 9], which are known to inhibit P450 14α-lanosterol demethylase activity (P450 51) in fungi [10], provides further indirect evidence that these organisms contain P450.

However, in African trypanosomes and *Crithidia* spp., neither P450-dependent enzyme activities nor P450 spectra have been reported. A carbon monoxide-binding pigment (λ_{max} 415–420 nm) has been reported in CO-reduced difference spectra in many trypanosomatids [11–14] and has been generally ascribed to cytochrome *o*, a terminal oxidase thought to account for cyanide-insensitive respiration in mitochondrial electron transport [15]. However, in the absence of convincing photochemical action spectra, the role of cytochrome *o* as a terminal oxidase remains open to question. Indeed, in a critical review [15], Hill has suggested that the cytochrome *o* spectrum could, in fact, be due to the cytochrome P450 degradation product cytochrome P420.

This paper describes investigations into the presence of cytochrome P450 mixed function oxidases in trypanosomatids. Representatives of the three major trypanosomal diseases of humans and domestic animals (*T. b. brucei*, *T. cruzi* and *L. donovani*), and the insect parasite *C. fasciculata* were all found to contain cytochrome P450-dependent activities. Cytochrome P450 spectra were also detected in *C. fasciculata* and *T. b. brucei*. As the anti-trypanosomal agent pentamidine is known to be metabolized by P450s in rats [16–18], the potential for metabolism of this compound by parasites was also investigated.

MATERIALS AND METHODS

Chemicals

Substrates, cofactors and inhibitors for cytochrome P450 reactions were obtained from the Sigma Chemical Co. (Gillingham, U.K.), and heptane sulfonate, carbon monoxide and tetramethylammonium chloride from the Aldrich Chemical Co. (Gillingham, U.K.). [^{3}H]Pentamidine (38.4 Ci/mmol) was prepared by Amersham (Amersham, U.K.) using [1,5-bis(2'-bromo-4'-amidinophenoxy)-pentane] supplied by the May and Baker Co. (now Rhone-Poulenc, Dagenham, U.K.). Ketoconazole was obtained from ICN (High Wycombe, U.K.). HPLC grade acetonitrile was acquired from BDH (Poole, U.K.), and all water was filtered and deionized by a Waters Milli-Q50 system (Watford, U.K.).

Parasites

T. b. brucei S427/118 (MiTat 1.5) trypomastigotes

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[19] were maintained in male Sprague–Dawley rats (HA Tuck, Essex, U.K.) and purified from blood by DEAE cellulose chromatography [20]. Heparinized whole rat blood was removed from uninfected animals for control experiments.

All other cloned organisms were cultured at 28°. *C. fasciculata* clone HS6 was grown in an undefined medium containing 5 mg/mL yeast extract (Difco, East Molesey, U.K.), 4 mg/mL tryptone (Difco), 15 mg/mL sucrose, 5 mg/mL Tween 80, 2.5 μL/mL triethanolamine and 50 μg/mL hemin [21]. *T. cruzi* X10/6 epimastigotes [22] were grown in RPMI 1640 medium (Gibco, Uxbridge, U.K.) containing 4.76 mg/mL *N*-2-hydroxyethyl-piperazine-*N'*-3-ethanesulfonic acid (Sigma), 4.90 mg/mL trypticase (Becton Dickinson, Oxford, U.K.), 20 μg/mL hemin, 10% heat-inactivated fetal calf serum (Gibco) and 100 U/mL penicillin/0.10 mg/mL streptomycin. *L. donovani* LV9 (MHOM/ET/67/HU3) promastigotes were grown in Grace's medium (Gibco) containing 20 μg/mL hemin, 10% heat-inactivated fetal calf serum and 100 U/mL penicillin/0.10 mg/mL streptomycin. Unless otherwise specified, cultured cells were grown to late log/early stationary phase before harvesting.

Preparation of subcellular homogenates

Rat liver microsomes were prepared from male Sprague–Dawley rats kept in cages containing sawdust and paper bedding and fed on RNM#1 expanded rat chow (BP Nutrition, Witham, U.K.). Livers were excised, rinsed with ice-cold 50 mM (K+) phosphate buffer, pH 7.4, minced with scissors and homogenized in two volumes of phosphate buffer or 50 mM phosphate buffer pH 7.4 containing 20% (v/v) glycerol, 1 mM ethylenediamine tetraacetic acid, 50 μM phenylmethanesulfonylfluoride, 5.5 mM phenanthroline, 50 μg/mL soybean trypsin inhibitor, 40 μg/mL aprotinin and 20 μg/mL leupeptin (buffer A) using a glass tube/Teflon pestle homogenizer (Jencons Scientific, Leighton Buzzard, U.K.). The microsomal fraction was isolated by differential centrifugation at 10,500 *g* for 15 min and 105,000 *g* for 60 min, and resuspended in buffer A or phosphate buffer. All microsomal samples were stored at −70°.

Post-mitochondrial supernatants were prepared using uninfected whole rat blood, or pelleted *T. b. brucei*, *T. cruzi*, *L. donovani* or *C. fasciculata* as described previously.* Full subcellular fractionation of *C. fasciculata* was performed essentially as described previously [23]. Pelleted *C. fasciculata* (8.0×10^9 cells) were washed and resuspended in 11 mL 0.32 M sucrose/25 mM Tris–HCl/pH 7.8 (buffer B) and transferred to an ice-cold mortar. Approximately 1 g of silicon carbide (BDH) was added and the cells ground until >95% of the cells were disrupted as judged by light microscopy. The homogenate was centrifuged for 3 min at 100 *g* to remove the silicon carbide. The supernatant was then centrifuged sequentially at 1000 *g* for 10 min, 14,500 *g* for 10 min and 105,000 *g* for 60 min. The pellets from each step were resuspended in 10 mL buffer B and stored at −20°. The 1000 *g* pellet consisted of nuclei and cell debris, the 14,500 *g* pellet of mitochondria and other large particles, the 105,000 *g* pellet of microsomes and other small particles, and the 105,000 *g* supernatant of cytosol.

Enzyme assays

Dealkylase activities. Rat liver microsomes (0.5 mL) in buffer A or trypanosomatid post-mitochondrial supernatant were mixed with 0.5 mL cofactor solution (2 mg/mL NADPH, 1.3 mg/mL glucose-6-phosphate and 0.1 U/mL glucose-6-phosphate dehydrogenase in phosphate buffer), and 10 μL of 100 mM ethoxycoumarin, 50 mM ethoxyresorufin or 50 mM pentoxyresorufin (all in methanol). For control incubations, 0.5 mL of phosphate buffer was added in place of the cofactor solution. All enzymatic rates were corrected for enzymatic activity in the absence of added cofactor solution. Non-enzymatic deethylation was less than 10% of the enzymatic rates. All samples were incubated for 60 min at 37° (rat liver or *T. b. brucei*) or 28° (*T. cruzi*, *L. donovani* or *C. fasciculata*). To inhibit P450 activity, carbon monoxide was gently bubbled into samples for 30 sec prior to incubation, or a final concentration of 1 mM proadifen, 2 mM metyrapone or 1 mM ketoconazole was added immediately before incubation. The amount of product formed was measured as outlined in Ref. 24. For each sample, 0.8 mL was transferred to a glass tube and 0.1 mL 2 N HCl and 2 mL chloroform were added. The samples were extracted, and 1.0 mL of chloroform was transferred to a new tube and back extracted with 2.5 mL of 30 mM sodium tetraborate, pH 9.2. The aqueous layer was then assayed using a Perkin–Elmer 3000 fluorescence spectrophotometer set at the excitation/emission pairing of 338/458 nm for ethoxycoumarin and 530/585 nm for the alkoxyresorufins. 7-Hydroxycoumarin and resorufin were used to construct standard curves for quantification.

Pentamidine hydroxylase. [^{3}H]Pentamidine (1 mL, 24.39 μCi/mL in phosphate buffer) was added to 1.0 mL of rat liver microsomes in phosphate buffer or trypanosomatid post-mitochondrial supernatant, and 1.0 mL of phosphate buffer or cofactor solution (as above). All samples were then incubated as described above. A portion of each incubation was analysed for pentamidine metabolites as described in Ref. 17. The system utilized was a Beckman 114M HPLC (High Wycombe, U.K.) equipped with a System Gold operating system, a model 506 autosampler and a model 167 variable wavelength spectrophotometer set at 265 nm. Fractions were collected every 0.5 min and radioactivity determined by liquid scintillation using a Beckman LS6000LL scintillation counter.

Other assays. Acid phosphatase [25] was measured fluorometrically by using 4-methylumbelliferylphosphate, L-alanine aminotransferase and isocitrate dehydrogenase as described previously [26, 27]. Cytochrome P450 was assayed by difference spectrophotometry of CO-reduced trypanosomatid post-mitochondrial supernatants using dithionite or NADPH as the reducing agent, and extinction coefficients of 91 and 110 $mM^{-1} cm^{-1}$ for P450 and P420, respectively [28]. Protein concentration was

* Berger, Carter and Fairlamb, *Acta Tropica*, in press.

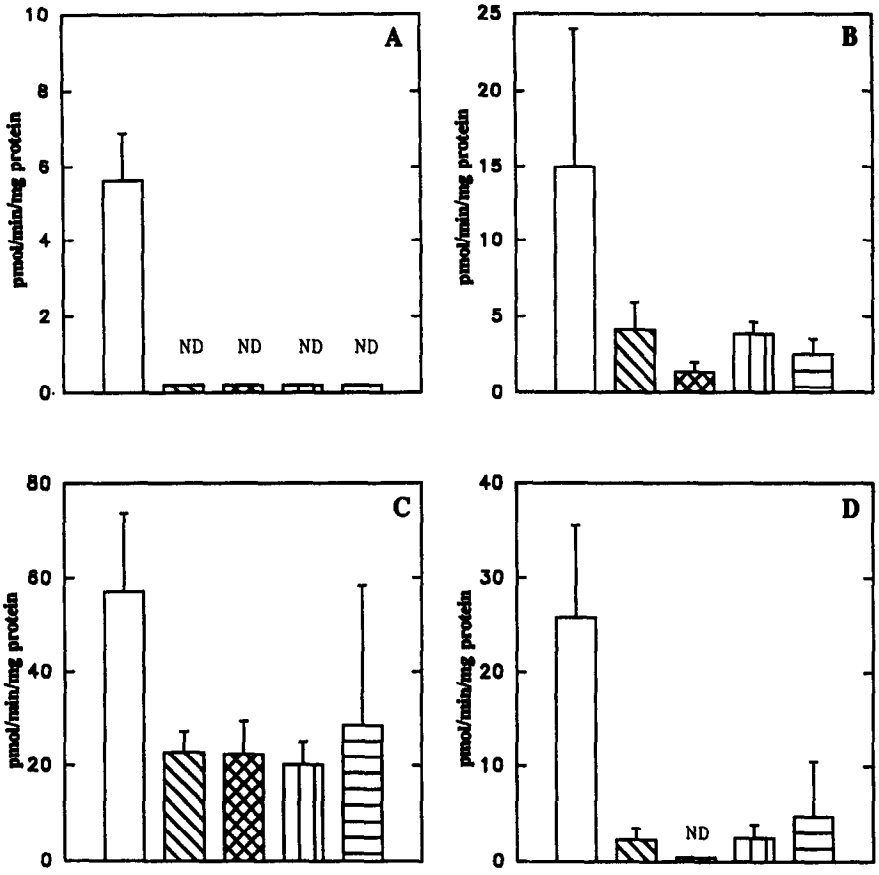

Fig. 1. Ethoxycoumarin deethylase activity of trypanosomatid post-mitochondrial supernatants. (A) *C. fasciculata*, (B) *T. b. brucei*, (C) *T. cruzi* and (D) *L. donovani* supernatants prepared, incubated with 100 μM ethoxycoumarin and assayed for the formation of hydroxycoumarin as described in Materials and Methods. In each plot are: supernatant plus cofactor solution without inhibitors (open bars), or with carbon monoxide (diagonal bars), 1 mM proadifen (cross-hatched bars), 2 mM metyrapone (vertical bars) or 1 mM ketoconazole (horizontal bars). ND, no detectable activity. For the *C. fasciculata* extracts in (A), the bars represent the mean ± SEM of 11 replicates for the incubations without inhibitors, and 4 for the incubations containing inhibitors. Each of the P450 inhibitors significantly decreases the deethylase activity (*t*-test; $P \leq 0.02$). For the other trypanosomatids (B–D), each bar represents the mean ± SD of two separate experiments. The value for rat liver microsomal ethoxycoumarin deethylase was 152 ± 46 pmol/min/mg protein (N = 2), and the addition of carbon monoxide, 1 mM proadifen, 2 mM metyrapone and 1 mM ketoconazole inhibited this activity 62%, 33%, 40% and 83%, respectively.

measured using the Bio-Rad protein assay system (Bio-Rad, Hemel Hempstead, U.K.).

RESULTS

Cytochrome P450-dependent enzyme activities

As sonication of cells had a deleterious effect on P450, and homogenization procedures using a glass tube/Teflon pestle system were not able to disrupt the trypanosomatids, it was necessary to utilize other homogenization techniques. Agitation of thick cell suspensions with glass beads (200–300 μm), often used to disrupt yeast cells [29], provided acceptable homogenates. Incubation of trypanosomatid post-mitochondrial supernatants with 7-ethoxycoumarin, known in rats to be catalysed by P450 1A1 [30], demonstrated that the fractions could metabolize the deethylation of the compound at approximately 10–30% of the rate catalysed by rat liver microsomes (Fig. 1). Since the trypanosomatid extracts are only partially purified, the trypanosome activity may be comparable to or greater than that for rat liver. Initial experiments showed that this activity was dependent on the presence of an NADPH generating system, was linear with respect to time up to 1 hr and was proportional to added protein up to 1 mg/mL (data not shown). Addition of carbon monoxide, proadifen and metyrapone, all specific P450 inhibitors, greatly decreased the rate of deethylation, strongly suggesting that P450 is present in all four trypanosomatid species. As *T. b. brucei* was isolated from rat blood, it was possible that blood cell contamination could be the source of ethoxycoumarin deethylase activity. Therefore, uninfected whole rat blood and a post-mitochondrial supernatant prepared

Table 1. Ethoxycoumarin deethylase activity of bloodstream *T. brucei* and uninfected rat blood

Sample	Protein (mg/mL)	Ethoxycoumarin deethylase (pmol/min/mg protein)
T. brucei S9	1.95	23.94
Whole blood S9	18.03	3.21
Whole blood*	46.66	0.91

* 8.43×10^9 erythrocytes/mL.
S9, post-mitochondrial supernatant.

from rat blood were assayed for this activity (Table 1). It was found that it would require 3.4×10^9 red blood cells, or more than seven times the volume of *T. b. brucei* post-mitochondrial supernatant to account for the reaction rate seen with the trypanosome fractions. Thus, the deethylase activity seen with *T. b. brucei* supernatants is not due to contamination by rat blood cells.

Since *T. cruzi* and *Leishmania* spp. are known to contain ergosterol, and are sensitive to ketoconazole [9, 31], it is possible that the ethoxycoumarin deethylase may be a marker for lanosterol demethylation in trypanosomatids. Therefore, ketoconazole was added to post-mitochondrial supernatants in an attempt to inhibit this activity (Fig. 1). The addition of 1 mM ketoconazole did, in fact, lead to inhibition of the deethylase activity in the trypanosomatid supernatants, but also inhibited the same activity in rat liver microsomes, where there is no known lanosterol demethylase activity. When the final concentration of ketoconazole was lowered to 100 μM, there was little detectable inhibition in either liver microsomes or trypanosomatid supernatants (data not shown). Therefore, the inhibition of ethoxycoumarin deethylase activity observed at 1 mM is most likely due to a more generalized inhibition of P450s, and not due to specific inhibition of a lanosterol demethylase.

The post-mitochondrial supernatants were also found to catalyse the deethylation of ethoxyresorufin (Fig. 2A), another substrate which is metabolized by P450 1A1 in rats [32]. The level of activity for *C. fasciculata* and *T. b. brucei* was approximately 20% of that detected in rat liver microsomes, whereas *T. cruzi* and *L. donovani* catalysed ethoxyresorufin deethylation at a rate comparable to that found in the microsomes. Addition of carbon monoxide, 1 mM proadifen and 2 mM metyrapone inhibited this activity by >50% in all trypanosomatid supernatants (data not shown). The subcellular fractions were also incubated with pentoxyresorufin (Fig. 2B), which is metabolized by P450 2B in rats [32], but none of the trypanosomatid extracts contained any detectable activity.

Pentamidine metabolism

To examine whether *C. fasciculata*, *T. cruzi* or *L. donovani* post-mitochondrial supernatants could metabolize pentamidine, radiolabeled drug was incubated with the subcellular fractions for 60 min and then analysed by HPLC. In all cases, the trypanosomatid fractions were unable to hydroxylate pentamidine to known metabolites [16–18] under conditions where rat liver microsomes metabolized >5% of the compound. Figure 3 shows the results obtained for *C. fasciculata*, which are markedly similar to those obtained for *T. cruzi* and *L. donovani* (not shown). A number of minor unidentified peaks were produced by the trypanosomatid fractions (e.g. 37, 48 and 59 min), which did not co-elute with any known compounds.

Spectral analyses

While numerous protease inhibitors were added

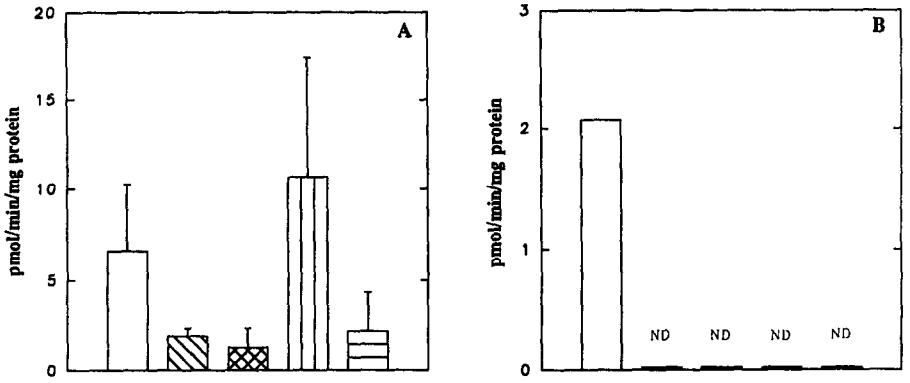

Fig. 2. The metabolism of alkoxyresorufins by trypanosomatid post-mitochondrial supernatants. Rat liver microsomes and trypanosomatid supernatants were prepared, incubated with 50 μM ethoxyresorufin or pentoxyresorufin, and assayed for the formation of resorufin as described in Materials and Methods. (A) Ethoxyresorufin deethylase activity and (B) pentoxyresorufin depentylase activity of (from left to right): rat liver microsomes (open bars), *C. fasciculata* post-mitochondrial supernatants (diagonal bars), *T. b. brucei* supernatants (cross-hatched bars), *T. cruzi* supernatants (vertical bars) and *L. donovani* supernatants (horizontal bars). The bars in (A) are the mean ± SD of two separate experiments, and those in (B) represent a single experiment. ND, no detectable activity.

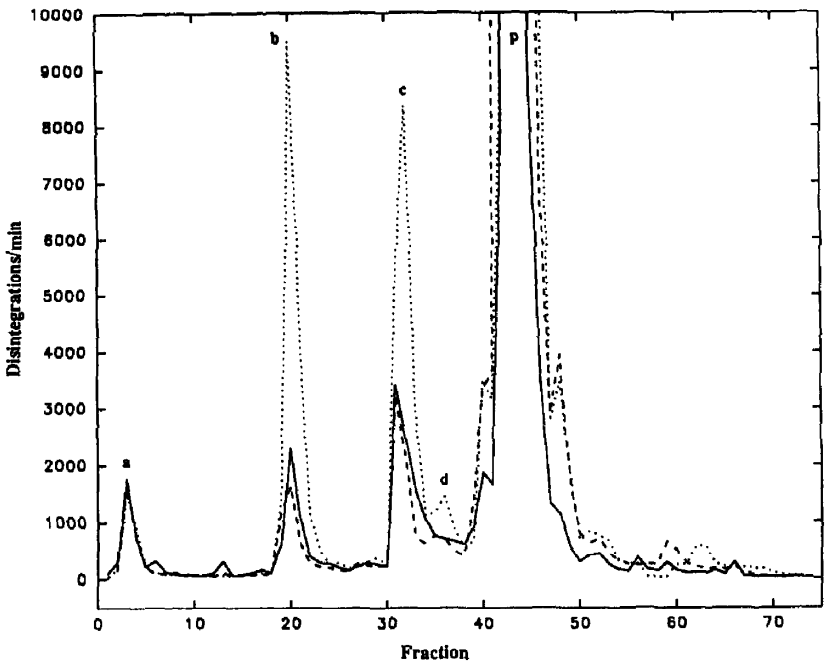

Fig. 3. The metabolism of [^{3}H]pentamidine by *C. fasciculata* post-mitochondrial supernatants and rat liver microsomes. [^{3}H]Pentamidine was incubated with P450 cofactor and buffer A (solid line), *C. fasciculata* supernatant (dashed line) or rat liver microsomes (dotted line) for 60 min before separation by HPLC and quantitation of radiolabel as described in Materials and Methods. The labeled peaks are: (a) a constant impurity peak (most likely ^3H_2O), (b) an impurity peak and the pentamidine metabolite *para*-hydroxybenzamidine, (c) an impurity peak and the pentamidine metabolites 5-(4'-amidinophenoxy)pentanoic acid and 5-(4'-amidinophenoxy)-1-pentanol, (d) the pentamidine metabolites 2- and 3-hydroxypentamidine, and (p) pentamidine. The concentration of protein was 2.59 mg/mL for the rat liver microsomes and 2.73 mg/mL for the *C. fasciculata* supernatant. A total of 0.163 μCi was injected on the HPLC column.

to the homogenization buffer to prevent deactivation of P450, the presence of these modifiers interfered with the spectral measurement of cytochromes. Therefore, for spectral analyses, cells were homogenized in 50 mM phosphate buffer containing 20% glycerol and 1 mM EDTA. Post-mitochondrial supernatants prepared from *C. fasciculata* were found to contain major 420 nm and minor 450 nm peaks after CO-dithionite difference spectroscopy (Fig. 4A). Samples reduced with NADPH gave similar spectra (Fig. 4B). The specific content of P450 was found to be 9.6 ± 2.5 (N = 4) pmol/mg protein, and varied considerably, most likely due to the degree of conversion to cytochrome P420. If the peak at 420 nM is assumed to be solely due to the presence of P420, then the P420 content of the cells was 99.4 ± 32.4 (N = 4) pmol/mg protein.

Spectra containing pronounced 420 nm and lesser 450 nm peaks were also obtained from bloodstream *T. b. brucei* post-mitochondrial supernatants (Fig. 5). This 450 nm peak was smaller than that detected in *C. fasciculata* fractions, and corresponded to 6.30 pmol/mg protein. As the *T. b. brucei* were isolated from rat blood, the 420 nm peak (which would be equivalent to 102 pmol/mg protein P420) could be due to small amounts of contaminating hemoglobin. Control experiments using uninfected rat blood indicated that it would require 3×10^5 contaminating erythrocytes (one erythrocyte per 10^5 trypanosomes) to produce a 420 nm carbon monoxide difference peak of equivalent size. Thus, given the problems of detecting such trace contamination, it is difficult to calculate the maximum potential P450 levels in bloodstream *T. b. brucei*.

Subcellular fractionation of C. fasciculata

Since *C. fasciculata* can be grown in the total absence of hemoglobin, and contains ethoxycoumarin deethylase activity and P450 spectra, this organism was fractionated to determine where the P450-dependent activity was localized. The cells, in early or late log phase, were ground with silicon carbide and than subjected to differential centrifugation to produce fractions enriched in nuclei and cell debris, mitochondria and large organelles, microsomes and small organelles, and cytosol. Each of these fractions was assayed for ethoxycoumarin deethylase activity and also for alanine aminotransferase (cytosol), acid phosphatase (lysosomes) and isocitrate dehydrogenase (mitochondria) (Fig. 6). The deethylase activity was located primarily in the microsomal fraction, with some activity detected in the mitochondrial fraction possibly due to microsomes trapped in that pellet. The control assays for cytosol, lysosomes and mitochondria show a distribution consistent with previous findings for *T. b. brucei*

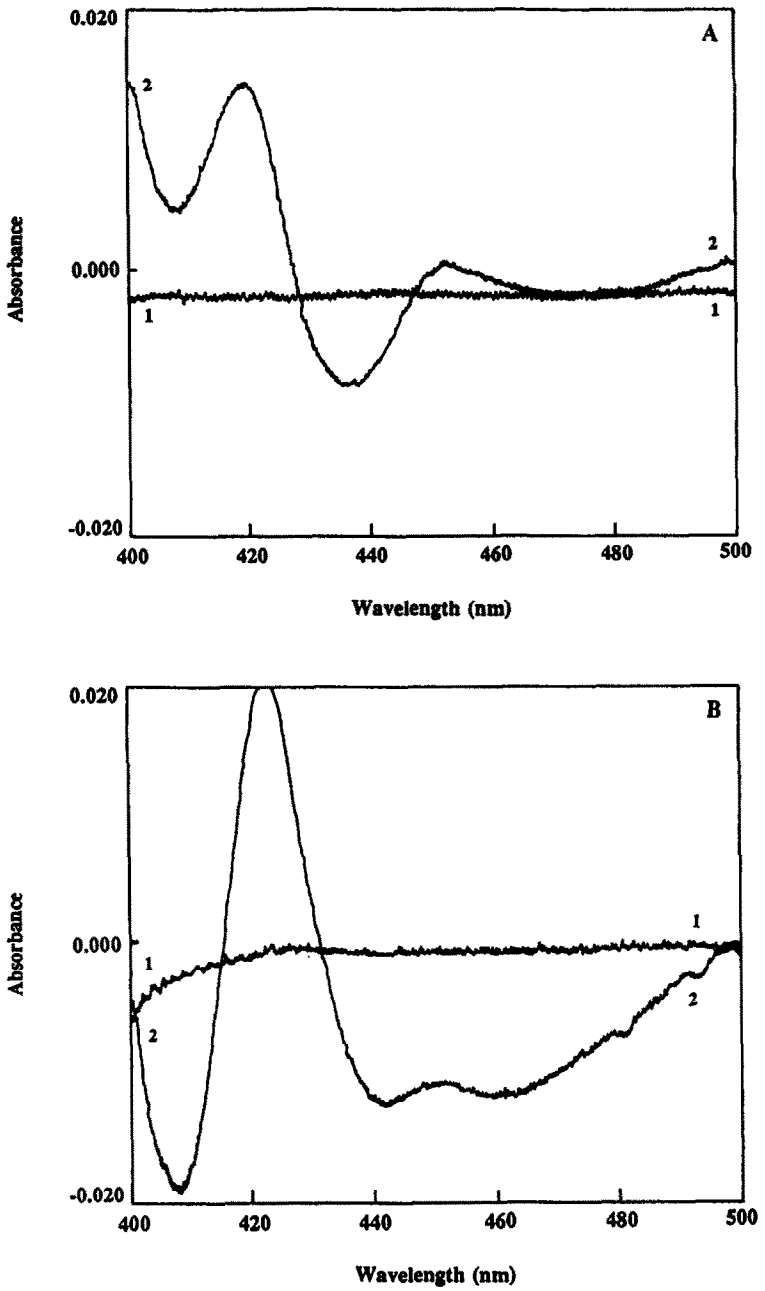

Fig. 4. Carbon monoxide difference spectrum of a *C. fasciculata* post-mitochondrial supernatant. (A) Samples reduced with sodium dithionite and (B) samples reduced with NADPH. The lines labeled (1) are reduced minus reduced baselines and (2) carbon monoxide reduced minus reduced difference spectra. The peak at 450 nm has a maximum at 453 nm.

[23, 33] suggesting that the majority of the P450-dependent deethylase in *C. fasciculata* is located in the microsomes. Hemoproteins producing a CO-difference peak at 420 nm were detected in the microsomal and mitochondrial fractions (not shown), but it was not possible to determine whether these peaks were due to cytochrome P420 or cytochrome *o*.

DISCUSSION

The results of the present studies show that *T. b. brucei*, *T. cruzi*, *L. donovani* and *C. fasciculata* all contain deethylase activities that have the characteristics of being catalysed by the cytochromes P450. First, the deethylase activities in all four species were inhibited by the addition of the specific

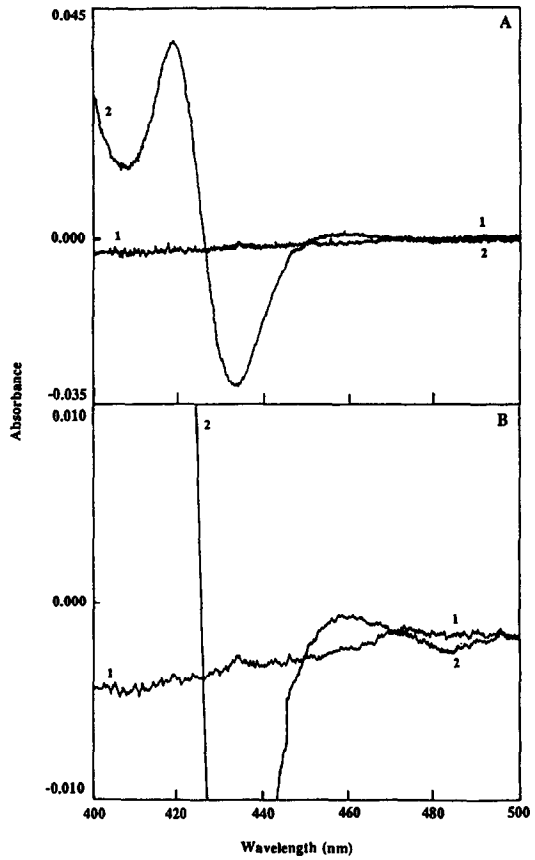

Fig. 5. Carbon monoxide difference spectrum of a *T. b. brucei* post-mitochondrial supernatant. (A) and (B) are the same sample at two different absorbance ranges. The line labeled (1) is a dithionite reduced minus dithionite reduced baseline and (2) a carbon monoxide-dithionite reduced minus dithionite reduced difference spectrum. The peak at 450 nm has a maximum at 457 nm.

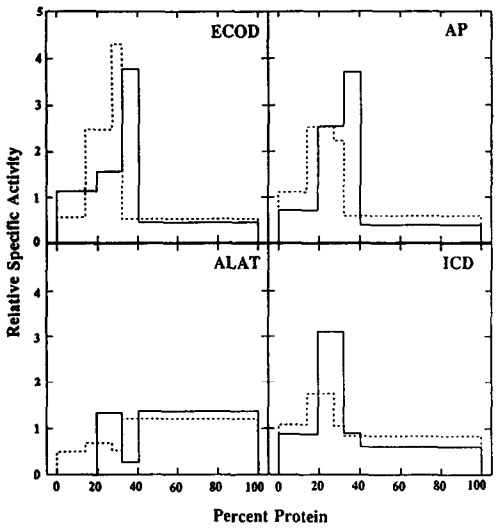

Fig. 6. The subcellular distribution of enzyme activities in *C. fasciculata*. In each plot the abscissa represents relative specific activity (% total act./% total protein), and the ordinate cumulative % protein (from left to right: nuclei, mitochondria, microsomes and cytosol). The solid line represents fractions isolated from late log/early stationary phase cells and the dashed line those from early log phase cells. ECOD, ethoxycoumarin deethylase activity; AP, acid phosphatase activity; ALAT, alanine aminotransferase activity; ICD, isocitrate dehydrogenase activity.

cytochrome P450 inhibitors carbon monoxide, proadifen and metyrapone. Second, *C. fasciculata* and *T. b. brucei* were found to produce typical cytochrome P450 CO-difference spectra on reduction with dithionite or NADPH. Finally, the ethoxycoumarin deethylase activity was found to be enriched in the microsomal fraction prepared from *C. fasciculata*. In the case of *T. cruzi*, these results confirm the previous investigations of Agosin *et al.* [3–5] and, for the other three organisms, provide the first evidence for the presence of cytochrome P450s. The reason for the 2–10-fold higher activity in the animal parasites than in *C. fasciculata* is not known.

The specific isozyme(s) of P450 being detected are unknown, but both ethoxycoumarin and ethoxyresorufin deethylase activities are known to be catalysed by P450 1A1 in rats and other mammals [30, 32]. While Agosin *et al.* [3] demonstrated that *T. cruzi* P450 content was phenobarbital inducible and Julistriono and Briand [34] that *Euglena gracilis* P450 is ethanol inducible, attempts to increase the P450 content of *C. fasciculata* with phenobarbital or ethanol have not been successful (data not shown). P450 1A1 inducers, such as 3-methylcholanthrene and β-naphthoflavone, have not yet been tested against cultures of trypanosomatids due to their very poor water solubility. All the organisms tested were unable to metabolize pentoxyresorufin, which is catalysed by P450 2B in rats [32]. The low amount of pentoxyresorufin deethylase activity reported here for rat liver microsomes is consistent with the findings of Burke *et al.* [32], which showed that this activity is very low when the microsomes have not been pre-induced with phenobarbital. The effect of phenobarbital on trypanosomal pentoxyresorufin depentylase activity has not been examined to date.

In addition, *T. cruzi*, *L. donovani* and *C. fasciculata* were unable to catalyse the hydroxylation of pentamidine, which is identical to results found previously for pentamidine-sensitive and -resistant *T. b. brucei*.* Therefore, neither the inherent resistance of *T. cruzi* to pentamidine nor the susceptibility of *L. donovani* is due to metabolism of the compound by the parasite. It would appear that trypanosomatids do not contain the same isozyme(s) of P450 responsible for the metabolism of pentamidine in rat liver [16–18].

One difficulty in studying the P450 content of trypanosomatids is the instability of the intact enzyme, as almost all of the detectable hemoprotein is in the form of P420. This phenomenon was also

* Berger, Carter and Fairlamb, *Acta Tropica*, in press.

noted by Agosin *et al.* [5] for the P450 present in *T. cruzi*. One potential source of interference is cytochrome *o*, which has been demonstrated by photochemical action spectra to act as a terminal oxidase in *T. mega* and *L. tarentolae* [14]. However, similar experiments performed on *C. fasciculata* were negative [35], leaving open the question as to whether cytochrome *o* exists in this organism. The possible presence of cytochrome *o* in *C. fasciculata* and contaminating hemoglobin in bloodstream *T. b. brucei* preparations complicates the quantification of P420. However, as neither of these two contaminating pigments yields a 450 nm CO-difference peak, the detection of such a peak in trypanosomatid extracts is indicative of the presence of intact P450. Due to the very small amount of intact P450 detected in the trypanosomatids, definitive proof for the existence of the enzyme(s) awaits the successful cloning and expression of the gene(s).

While the exact P450(s) in the trypanosomatids remains unknown at this point, further study may identify the isoenzymes present. It would then be possible to determine what role these enzymes play in cell metabolism and, potentially, to exploit these pathways therapeutically.

Acknowledgements—This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Wellcome Trust (A.H.F.), and the NATO Science Fellowship Program (B.J.B.).

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