Evidence for the presence of active cytochrome P450 systems in *Schistosoma mansoni* and *Schistosoma haematobium* adult worms

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Abstract Extracts of the adult worms of both *Schistosoma mansoni* and *Schistosoma haematobium* can metabolise some typical P450 substrates but to differing degrees. *S. mansoni* worm extracts displayed a ~12-fold higher specific activity for an aminopyrine substrate than rat liver microsomes. At 4 mM substrate concentration the demethylation reaction with *N*-nitrosodimethylamine (NDMA) (5 nmol HCHO/mg protein/min) was only half that of rat liver microsomes, whereas in extracts of *S. haematobium*, no detectable activity was found towards NDMA. Using ethylmorphine as substrate the demethylation activity of *S. mansoni* extracts (1.82 nmol HCHO/mg protein/min) was 5.5-fold lower than that of rat liver microsomes. Benzphetamine demethylase activity was also readily detectable in *S. mansoni* worm extracts at 6.79 nmol HCHO/mg protein/min compared with 10.20 nmol HCHO/mg protein/min in the case of rat liver microsomes. When aniline was used as substrate, surprisingly, no activity was found in worm extracts of either *S. mansoni* or *S. haematobium*, whereas rat liver microsomes showed high activity towards this amine. The anti-P450 2E1 and 2B1/2 cross-reacted with both worm homogenates and gave a specific band corresponding to a protein of molecular weight of ~50.0 kDa. A study with anti-P450 IVA antibody revealed that while this protein was strongly expressed in *S. haematobium* worm extracts, no immunoreactivity was observed with extracts of *S. mansoni*. Immunoblotting analyses with anti-P450 IIIA and P450 1A1 did not detect immunoreactive protein in either *S. mansoni* or *S. haematobium*. **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Cytochrome P450 reductase; CYP2E1; CYP1A1/A2; CYP2B1/B2; Aniline hydroxylase; Demethylation reaction; *N*-Nitrosodimethylamine; Schistosome

1. Introduction

Adult schistosome worms inhabit host blood vessels and have evolved a number of strategies to survive in this aerobic environment, rendering them invulnerable to the host immune system. Among the enzymes that play an important role in parasite survival is the phase II drug-metabolising enzyme glutathione *S*-transferase which is involved in the detoxification of xenobiotics, including the antischistosomal drug dichlorvos [1]. The parasite's anti-oxidant enzyme system may also play a key role in allowing adult *Schistosoma mansoni* and *Schistosoma haematobium* worms to tolerate oxyradical attack from activated macrophages [2] and granulocytes [3]. Investigation of the metabolising systems present in adult worms has shown that superoxide dismutase, glutathione peroxidase, glutathione *S*-transferase, tripeptide glutathione, and a cytochrome *c* peroxidase-linked activity are present [3], but so far cytochrome (CYP) P450 activities have not been reported. CYP-P450 proteins are the products of a gene superfamily of haem-binding monooxygenases found in many diverse species [4]. They are well documented, particularly in vertebrates, but little is known of P450 genes in blood parasites of the genus *Schistosoma*.

Since the adult *S. mansoni* worms live in the inferior mesenteric venous system where they are potentially exposed to the same toxic substances that reach the liver, a parasite P450 system would be expected to play a role in the survival of the parasite within the host environment. In this present study a functional P450 system was identified in adult worms of both *S. mansoni* and *S. haematobium*.

2. Materials and methods

2.1. Schistosome worms

Schistosomes used in this study were as follows: adult worms of *S. mansoni* (Puerto Rico isolate) and *S. haematobium* were obtained from life cycles maintained, respectively, in TO mice and Syrian golden hamsters at the School of Biological Science, University of North Wales, Bangor, UK.

2.2. Lymphocytes

Whole blood was collected from BDF1 (C57BL/DBA) mice under terminal anaesthesia by cardiac puncture into universal vials containing 200 μl 0.5 M EDTA. Vials were centrifuged at 800×*g* for 10 min, the buffy coat was harvested and then resuspended in 10 ml of 0.16 M ammonium chloride. The resuspended white cells were mixed to lyse any residual erythrocytes and centrifuged immediately at 800×*g* for 8 min. The pellet was then resuspended in ice-cold 100 mM potassium phosphate buffer, pH 7.4 and homogenised using a glass homogeniser.

2.3. Antibodies

A mouse monoclonal anti-rat P450 2E1, 1-98-1, was a gift from Prof. H. Gelboin and Dr Kris Krausz, Laboratory of Molecular Carcinogenesis, National Institute of Health, Bethesda, MD, USA. Anti-P450s 2B1/2, 1A1, IIIA and IVA (polyclonal anti-rat) were purchased from Amersham International (Amersham, UK).

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Abbreviations: CYP, cytochrome P450 protein; NDMA, *N*-nitrosodimethylamine

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2.4. Sample preparation

Outbred Wistar rat liver and hamster liver microsomes were prepared according to Mostafa et al. [5]. Livers were removed immediately after decapitation, chilled on ice, washed with 100 mM potassium phosphate buffer, pH 7.4 and homogenised in three volumes (w/v) of the same buffer. The crude homogenate was centrifuged at $10\,000\times g$ for 10 min and the supernatant fraction was further centrifuged at $100\,000\times g$ for 1 h, both at 4°C to yield the microsomal pellet. This was then resuspended in 100 mM potassium phosphate buffer pH 7.4 and used for assays of enzymatic activity and for immunoblotting studies of P450 proteins. Microsomes were also prepared from rats exposed to 5% (v/v) ethanol for 3 days or to 0.05% (w/v) phenobarbital for 1 week both via the drinking water to induce, respectively, CYP2E1 [6] or CYP2B1/2 [7]. Pregnenolone 16α-carbonitrile-, clofibrate- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced microsomes were purchased from Amersham International.

Preparation of worm extracts: male and female adult *S. mansoni* and *S. haematobium* worms were homogenised with ice-cold 100 mM potassium phosphate buffer, pH 7.4. The crude homogenates were centrifuged at $10\,000\times g$ for 10 min and the supernatant at $100\,000\times g$ for 1 h at 4°C exactly as described above for the liver microsomes. It was found that the P450 activity towards *N*-nitrosodimethylamine (NDMA) was four- to five-fold higher in the $10\,000\times g$ and this supernatant was used for all subsequent enzyme assays and immunoblotting analyses.

2.5. Functional assays of cytochrome P450

Aminopyrine, NDMA, ethylmorphine and benzphetamine demethylase activities were assayed according to El-Amri et al. [8] and Yang et al. [6] and the amount of formaldehyde formed was determined by the method of Nash [9]. The assay mixture (0.5 ml) contained 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM KCl, NADPH-generating system (0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, and 0.8 U glucose-6-phosphate dehydrogenase) and rat liver microsomes as a positive control or *Schistosoma* worm extract with one of the following substrates: 4 mM aminopyrine or NDMA and ethylmorphine (1 mM), or benzphetamine (2 mM). The reaction components were preincubated at 37°C prior to initiating the reaction by the addition of the relevant substrate and the reaction was carried out for 30 min; blanks were prepared by omitting the NADPH-generating system. A unit of activity was defined as 1 nmol HCHO/mg protein/min.

Dealkylation reactions (ethoxy- and pentoxyresorufin *O*-dealkylase activity) were carried out according to Wardlaw et al. [10] and were quantitated by measurement of increased resorufin fluorescence with time. Reactions were carried out in a total volume of 1.2 ml and microsomal protein or *Schistosoma* worm extracts were added to 0.1 M potassium phosphate buffer pH 7.6 together with 2 μM ethoxy or pentoxyresorufin (Sigma, Poole, UK). The substrate–microsome solution was then incubated for 2 min at 37°C prior to initiation of the reaction with 0.5 mM NADPH (Sigma). The dealkylation reaction was allowed to proceed for 10–15 min at 37°C and then was terminated by the addition of 2 ml of cold methanol. A unit of activity was defined as 1 pmol resorufin/mg protein/min.

Aniline hydroxylase activity was assayed according to Imai et al. [11] by following the formation of *p*-aminophenol from aniline. The reaction mixture contained 8 mM aniline, 0.32 mM NADP, 3 mM glucose 6-phosphate, 2.5 mM $MgCl_2$, 1.3 U of glucose-6-phosphate dehydrogenase, 8.0 mM nicotinamide, 100 mM Tris–HCl buffer, pH 8.0, and measured amounts of *S. mansoni* and *S. haematobium* worm extract; rat liver microsomes were also assayed as a positive control. Reactions were carried out for 30 min at 37°C. A unit of activity was defined as 1 μmol *p*-aminophenol liberated/mg protein/min.

NADPH-cytochrome P450 reductase was measured according to Yasukochi et al. [12]. The assay mixture contained 2 mM NADPH, 0.8 mM cytochrome *c* and 375 mM potassium phosphate buffer pH 7.7 and reactions were performed at 37°C for 10 min. The activity was calculated using the molar absorption coefficient of 550 nm = 21 mM^{-1} cm^{-1}. A unit of reductase activity is 1 μmol of cytochrome *c* reduced/μg protein/min.

2.6. Protein analysis

Total protein concentrations of schistosome extracts and of rat liver microsomes were assayed by the method of Bradford [13] using a calibration curve established with bovine serum albumin as a standard. Proteins in these preparations were analysed by SDS–PAGE on 10% gels according to the method of Laemmli [14]. The gel proteins were then transferred by electroblotting to a nitrocellulose membrane (High bond C-extra, Amersham) according to Towbin et al. [15]. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL Kit, Amersham International).

3. Results

3.1. Functional assays

In order to detect the presence of P450 protein and to define the common P450 subclasses in schistosomal worm extracts, different P450 substrates were used. Extracts of *S. mansoni* and *S. haematobium* showed detectable, protein concentration-dependent *N*-demethylation activity, which for *S. mansoni* was approximately 12-fold higher for aminopyrine demethylation than for rat liver microsomes (9.3 versus 0.76 nmol HCHO/mg protein/min). In the case of *S. haematobium*, the calculated specific activity was 2.58 nmol HCHO/mg protein/min, which is three times higher than that of rat liver microsomes and four times less than determined for *S. mansoni* extract (Table 1).

At 4 mM NDMA the specific activity of the demethylase activity for the *S. mansoni* extract (4.9 nmol HCHO/mg protein/min) was about half that of rat liver microsomes (7.6 nmol HCHO/mg protein/min) (Table 1). Surprisingly, extracts of *S. haematobium* displayed no NDMA demethylase activity when assayed under identical conditions.

The ethylmorphine deethylase activity of a *S. mansoni* worm extract showed a protein-dependent increase in activity which was 5.5-fold lower than that of rat liver microsomes. Benzphetamine demethylase activity was also readily detectable in the *S. mansoni* worm extract but was 1.5-fold lower

Table 1
Summary of the functional assays for P450 in *S. mansoni*, *S. haematobium* and acetone-induced rat liver microsomes (positive control)

Substrate used	Specific P450	Substrate concentration	Specific activity		
			S. mansoni	*S. haematobium*	Rat liver microsomes, acetone-induced
Aminopyrine[a]	2E1	4.0 mM	9.30	2.58	0.76
NDMA[a]	2E1	4.0 mM	4.97	N.D.	7.65
Ethylmorphine[a]	2E1	1.0 mM	1.82	–	9.95
Benzphetamine[a]	2E1	2.0 mM	6.79	–	10.20
Aniline[b]	2E1	8.0 mM	N.D.	N.D.	3.50
Pentoxyresorufin[c]	2B1 and 2B2	1.5 pM	70.32	–	22.20
Ethoxyresorufin[c]	1A1 and 1A2	2.0 pM	N.D.	N.D.	88.86

[a]For aminopyrine, NDMA, ethylmorphine, and benzphetamine, the specific activity is expressed as nmol HCHO/mg protein/min.
[b]For aniline hydroxylase the specific activity is expressed as μmol *p*-aminophenol liberated/mg protein/min.
[c]For pentoxy- and ethoxyresorufin, the specific activity is expressed as pmol resorufin/mg protein/min.
N.D.: not detected.

than that of rat liver microsomes (i.e. 6.79 versus 10.20 nmol HCHO/mg protein/min, Table 1).

Hydroxylase activity was not detectable using aniline as a substrate at 8 mM concentration, in either *S. mansoni* or *S. haematobium* worm extracts, whereas rat liver microsomes showed a relatively high hydroxylase activity (3.5 μmol aminophenol/mg protein/min) when assayed under identical conditions (Table 1).

The specific activity for pentoxyresorufin dealkylase activity for the *S. mansoni* worm extract was three-fold higher (70.32 versus 22.20 pmol resorufin/mg protein/min) than that of rat liver microsomes (Table 1), but showed no detectable ethoxyresorufin dealkylation when assayed at the same time as rat liver microsomes, which showed very high specific activity (88.86 pmol resorufin/mg protein/min).

NADPH-cytochrome P450 reductase was measured using an indirect method for detection of general broad-range P450 activity. Both *S. mansoni* and *S. haematobium* worm extracts showed clear evidence of P450 reductase activity that increased with protein concentration in the assay. This reductase activity was much lower than that of rat liver microsomes (10.0 μmol cytochrome *c* reduced/mg protein/min for *S. mansoni* versus 147.8 for rat liver microsomes). For the *S. haematobium* worm extract, P450 reductase activity was evident at 3.5 μmol cytochrome *c* reduced/mg protein/min.

3.2. Immunoreactivity

To complement the findings obtained using in vitro activity assays, different anti-P450 antibodies were used to detect the presence of P450 in *S. mansoni* and *S. haematobium* worm extracts employing SDS–PAGE. These results and the individual observations are detailed as follows. Anti-P450 2E1 cross-reacted with both worm extracts, giving in each case a specific band which corresponded to a protein with a molecular weight of ~52 000 Da and similar to the one observed in rat liver microsomes (Fig. 1A).

Immunoblotting analysis of both *S. mansoni* (Fig. 1B) and *S. haematobium* (Fig. 1C) worm extracts with anti-P450 2B1/2 revealed that both contained a protein that was strongly immunoreactive with this antibody and which also had a molecular weight of ~52 000 Da. As expected, uninduced rat liver microsomes and uninduced hamster liver microsomes (Fig. 1D) showed no cross-reactivity with this antibody (2B1/2).

Immunoblotting analysis with anti-P450 IA (Fig. 1E) antibody failed to detect immunoreactive protein in either *S. mansoni* or *S. haematobium* worm homogenates. Similarly no antigen was detected in either of these worm extracts when anti-P450 IIIA was used (Fig. 1F). Both proteins were assayed under conditions that readily allowed detection of the induced protein in rat liver microsomes (Fig. 1E,F).

No immunoreactivity was obtained with *S. mansoni* worm supernatant using anti-P450 IVA antibody (Fig. 1G) whereas strong cross-reactivity was obtained with *S. haematobium* worm supernatant (Fig. 1H), indicating that this form of P450 may be strongly expressed in *S. haematobium* adult worms. This implies a clear distinction in the expression profile of a P450 IVA-like protein between the two worm species. In each case a strong signal was detected in clofibrate-induced rat liver microsomes. The protein identified in the *S. haematobium* worm extracts appeared to be of lower molecular weight than the rat equivalent.

One potential concern relating to the analysis of enzymatic

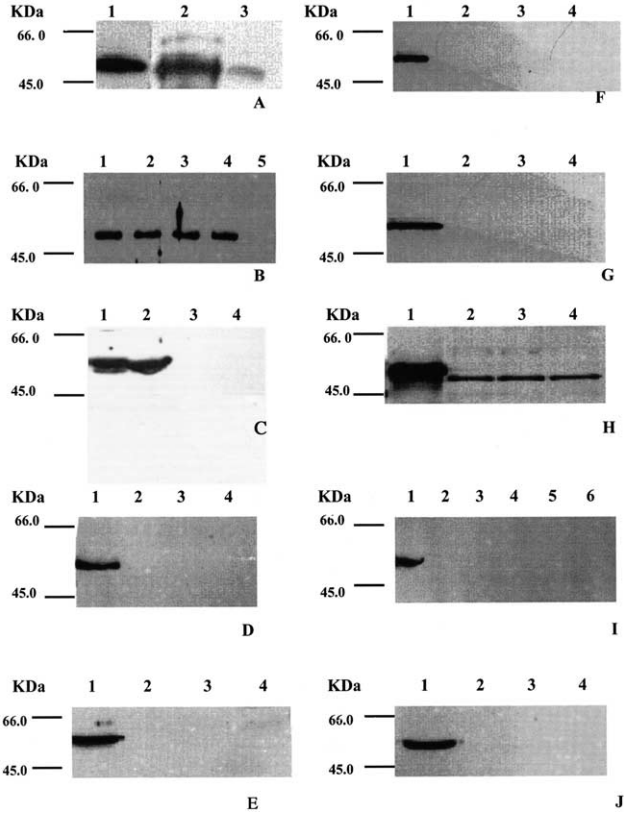

Fig. 1. Western blot analyses of adult schistosome worm homogenates and rodent liver microsomes (rlm) using anti-P450 proteins. A: Anti-P450 2EI: lanes 1–3, *S. haematobium, S. mansoni* and uninduced rat liver microsomes (rlm) (25 μg protein/lane), respectively. B: Anti-P450 2B1/2: lanes 1–4, *S. mansoni* and lane 5 uninduced rlm (25 μg protein/lane). C: Anti-P450 2B1/2: lanes 1 and 2, *S. haematobium*, lane 3, blank and lane 4, uninduced rlm (30 μg protein/lane). D: Anti-P450 2B1/2: lane 1, phenobarbital-induced rlm (5 μg protein), and lanes 2–4, uninduced hamster liver microsomes (25 μg protein/lane). E: Anti-P450 1A1: lane 1, TCDD-induced rlm (5 μg protein); lanes 2 and 4, *S. haematobium* and *S. mansoni* (30 μg protein/lane), respectively and lane 3, blank. F: Anti-P450 IIIA: lane 1, pregnenolone 16α-carbonitrile-induced rlm (5 μg protein); lanes 2 and 4, *S. haematobium* and *S. mansoni* (30 μg protein/lane), respectively and lane 3, blank. G: Anti-P450 IVA: lane 1, clofibrate-induced rlm (5 μg protein) and lanes 2–4, *S. mansoni* (25 μg protein/lane). H: Anti-P450 IVA: lane 1, clofibrate-induced rlm (5 μg protein) and lanes 2–4, *S. haematobium* (25 μg protein/lane). I: Anti-P450 2E1: lane 1, clofibrate-induced rlm, lanes 2 and 3, uninduced hamster liver microsomes and lanes 4–6, mouse lymphocytes (25 μg protein/lane). J: Anti-P450 2B1/2: lane 1, phenobarbital-induced rlm (5 μg protein), lane 2, uninduced hamster liver microsomes (25 μg protein), lane 3, blank and lane 4, mouse lymphocyte supernatant.

functions of protein expression in worms obtained by liver perfusion is that some or all of the activities assayed could be accounted for, either by contamination from ingested host white blood cells or by contamination from traces of host liver. Therefore, immunoblotting analyses of uninduced rat and hamster liver microsomes and lymphocyte extracts were carried out (Figs. 1I,J). In all cases no immunoreactive protein was obtained with the anti-rabbit P450 2B1/2 and anti-rat P450 2E1 antibodies that reacted strongly with both types of schistosomal worm homogenates. These results indicated that contamination from either the blood of the host organism or the liver was extremely unlikely to account for the data produced using worm extracts.

4. Discussion

Using a variety of P450 substrates in functional assays (summarised in Table 1) and immunoblotting with characterised anti-P450 proteins, it has been possible to identify P450 enzyme activities in extracts of either or both *S. mansoni* and *S. haematobium*. A strong P450 2EI activity was identified in *S. mansoni* that was higher or of a similar order to the specific activity of rat liver microsomes used as a positive control in this study (Table 1). For one substrate (aminopyrine) the *S. mansoni* activity was an order of magnitude higher than that of the mammalian preparation. The limited volume of extract available precluded a similar broad-spectrum study for *S. haematobium* but assays with aminopyrine revealed an activity greater than that with rat liver microsomes. Interestingly no detectable activity was found in *S. haematobium* extracts when assayed with NDMA as substrate, possibly suggesting a qualitative difference in gene regulation between the schistosome species or a very low level of expression in *S. haematobium*. Either or both of these possibilities might result from adaptation to their differing locations within the host's body.

NDMA has been shown to be an acute hepatotoxin and a potent carcinogen in many animal species and also potentially in humans [16,17]. In experiments with human liver microsomes, it has been demonstrated that cytochrome P450 2EI is the major enzyme responsible for the oxidative demethylation of NDMA at low substrate concentrations [18]. Moreover, P450 2E1 is considered to be a major catalyst responsible for the metabolism of many low molecular weight carcinogens and potentially toxic chemicals including ethanol, nitrosamines, halogenated alkanes and aromatic compounds [18–20]. These results using aminopyrine and NDMA as substrates indicated that worm homogenate from *S. mansoni* and to a lesser extent that of *S. haematobium* could metabolise these substrates, due to the presence of P450 enzymes which may be similar or identical to 2E1.

Using aniline, another potential P450 2E1 substrate, it was not possible to detect hydroxylase activity in extracts of either schistosomal species, suggesting that while a P450 2E1-like demethylase activity was present in both worm species, this did not extend to hydroxylase activity.

The presence of ethylmorphine- and benzphetamine-type dealkylase activities adds further support for the existence of a P450 system with proteins similar to 2EI in *S. mansoni* worm extracts. However, due to sample limitation, no data were obtained for this substrate with *S. haematobium*.

Investigation of possible P450 1A1/2 activity using ethoxyresorufin revealed that both *S. mansoni* and *S. haematobium* were apparently totally deficient in this activity under assay conditions which readily detected the same activity in induced rat liver microsomes. Wardlaw et al. [10] showed that the metabolism of ethoxyresorufin is associated with P450 1A1 and 1A2, with the 1A1 form metabolising ethoxyresorufin to a much greater extent than 1A2 when both P450s are present. P450 1A2 is a major constitutive enzyme in human liver and has been identified as a key factor in the bioactivation of a large number of environmental contaminants including aflatoxin, arylamines and dietary heterocyclic amines [21]. These data indicate P450 1A1/2, or a close homologue, may not be expressed in adult schistosomal worms. Alternatively, it is possible that this form of P450 may need to be induced with an appropriate inducing agent (such as TCDD or polyaromatic hydrocarbons) which are well known to upregulate mammalian P450 1A1/2 [22]. Additional possibilities are that other factors such as enzyme stability, the need for unique cofactors or the presence of inhibitors in the worm extract, somehow may have compromised these assays of activity. P450 1A1 has also been shown to be highly inducible in animals treated with chemical inducers such as polycyclic aromatic hydrocarbons [22]. The constitutive levels of both these P450 isozymes are low and normally represent less than 1% of the total P450 protein content of livers in untreated, male or female, rat liver.

The high assayable level of 2B1/2 activity in *S. mansoni* worms suggests that because the P450 form (2B1/2) is usually induced by administration of barbiturate compounds such as phenobarbital [7] or steroids such as dexamethasone [22], there may be P450 proteins that are uniquely expressed in these schistosomes. The pentoxyresorufin deethylase reaction is thought to be carried out by P450 2B1 and 2B2 and these data imply that an analogous system may operate in *S. mansoni* adult worms.

It has been reported that P450 IIIA can be induced by steroids such as dexamethasone or pregnenolone-16α-carbonitrile, as well as by phenobarbital and some antibiotics; members of this P450 family can also be developmentally regulated [23–26]. As this activity was not detected in worm extracts, it is not unreasonable to conclude that expression of P450 IIIA could either be controlled by a developmentally regulated switch or only be expressed in response to an appropriate inducing signal and that such a regulatory mechanism is shared by *S. mansoni* and *S. haematobium*.

The data produced in this study have not only demonstrated that schistosomes possess a range of different P450 activities, but suggest that differences in levels of expression of P450 proteins between the parasites and their host may be sufficient to allow new classes of anti-schistosomal drugs to be developed. The expression patterns of other P450 subclasses, their inducibility and their substrate specificities also need more rigorous investigation in future studies, but the present data clearly imply that this strategy may have some merit.

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