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INDUCTION OF HEPATIC CYTOCHROME P-450c-DEPENDENT MONOOXYGENASE ACTIVITIES BY DANTROLENE IN RAT

Z. JAYYOSI,* M. TOTIS,* H. SOUHAILI,* C. GOULON-GINET,† M. H. LIVERTOUX,* A. M. BATT* and G. SIEST*

*Centre du Médicament, U.A. C.N.R.S. 597, 30 rue Lionnois, 54000 Nancy, and †Laboratoire de Chimie Théorique, Faculté des Sciences, 54100 Vandoeuvre-les-Nancy, France

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Abstract—The effect of dantrolene sodium, a skeletal muscle relaxant, on drug metabolizing enzymes has been investigated after treatment of rats with a dose of 200 mg/kg for five days. We observed an induction of cytochrome P-450c and epoxide hydrolase in immunoassays and activities. An enhancement of the UDP-glucuronosyltransferase (GT1) activity was observed. We also reported a decrease of both liver cytochrome P-450 content and microsomal cytochrome P-450b dependent *N*-demethylation activities. On the other hand, the binding of dantrolene on microsomal cytochrome P-450 produced a type I difference spectrum, these data were obtained with liver microsomal cytochrome P-450c induced by 3-methylcholanthrene.

Dantrolene sodium, 1-{[5-(p-nitrophenol) furfuryl-idene] amino}-hydantoin sodium hydrate is a skeletal muscle relaxant used to decrease muscle spasticity [1]; it is also used in the treatment of the malignant hyperthermia (MH) reaction, in the prevention of this reaction in persons susceptible to be affected and to lower body temperature due to causes other than MH.

The use of dantrolene as a myorelaxant is limited by its side effect of hepatic damage, with a mortality rate of about 0.3% which is related to the long term dantrolene therapy, and to the drug dosage [2]. The mechanism involved in hepatic toxicity has not yet been clearly demonstrated, thus the metabolic pathways were a possible hypothesis. The metabolism of dantrolene has been reported [3] to proceed through both reductive and oxidative pathways (Fig. 1). The nitro group of dantrolene is reduced to amino dantrolene; in some mammals, including man, the amino dantrolene is acetylated. The oxidative pathway produces 5-hydroxydantrolene. Ellis *et al.* have shown that 5-hydroxydantrolene relaxes skeletal muscle as well as dantrolene and that aminodantrolene has minimal relaxing properties [4].

According to Arnold *et al.* [5], the reductive pathway which produces aminodantrolene could be the cause of the bioactivation of dantrolene. This may proceed through covalent binding of dantrolene or its metabolites to the protein. An inhibitory effect of dantrolene and aminodantrolene on some hepatic cytochrome P-450 dependent monooxygenase activities has been reported by Francis *et al.* [6]. It is characterized by a decrease in the liver cytochrome P-450 content and by an inhibition in the binding of ethylmorphine, a type I substrate, to liver microsomal cytochrome P-450. Nevertheless no toxic metabolite was specifically characterized. In the present work, we investigated the effects of an *in vivo* administration of dantrolene on rat liver microsomal cytochromes P-450. We took into account the existence of different cytochrome P-450 isozymes, especially cytochrome P-450c, which was not previously tested by others, although it was suspected to be involved in numerous toxic processes [7]. We also tested the interaction of dantrolene with these different isozymes of cytochrome P-450, using the difference spectrum method [8]. Moreover, we determined some phase II drug metabolizing enzyme activities like microsomal epoxide hydrolase and UDP-glucuronosyltransferase. Therefore we treated rats with 3-methylcholanthrene (3-MC),‡ phenobarbital (PB) or dantrolene.

MATERIALS AND METHODS

Chemicals. Sodium dantrolene was a generous gift from Oberval (Lyon, France), 3-MC was obtained from Sigma chemicals, PB was purchased from Fluka (Buchs, Switzerland). All other chemicals and biochemicals were of analytical grade or better. The purity of dantrolene was checked by NMR.

Animals. Male Sprague–Dawley rats (180–200 *g*) were obtained from Iffa Credo (L'Arbresle-France) and kept in the animal room at 20° on sow dust; rabbits (Fauve de Bourgogne) were from a local supplier.

Pretreatments. Sodium dantrolene, as a suspension in corn oil (200 mg/kg), was administered i.p. (1 ml) once daily for five days to male Sprague–Dawley rats [8]. The animals were decapitated 48 hr after the last injection. 3-MC in corn oil (80 mg/kg) was administered by a single i.p. injection and rats were decapitated 5 days later. Control rats received an equal volume of corn oil. PB sodium salt, in NaCl 0.9%

‡ Abbreviations used: EDTA, ethylene diamine tetra-acetic acid; DTT, dithiothreitol; PB, phenobarbital; 3-MC, 3-methylcholanthrene.

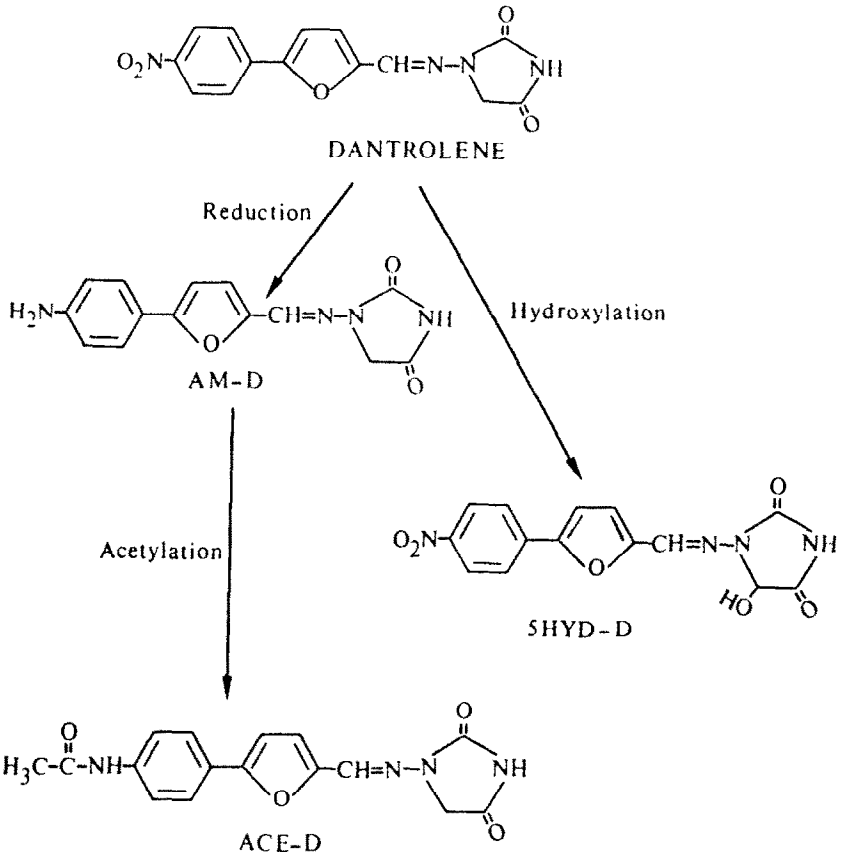

Fig. 1. Proposed metabolic scheme of dantrolene sodium, AM-D, aminodantrolene, 5HYD-D, 5-hydroxydantrolene, ACE-D, acetyldantrolene.

solution, was injected i.p. (1 ml), once daily at the dose of 80 mg/kg for five days; the animals were decapitated on the 6th day. During the experiments, rats were provided with UAR chow (Villemoisson sur Orge, F), and water *ad libitum*. All rats were starved 18 hr before decapitation.

Preparation of microsomes. After decapitation, the livers were rapidly removed and placed on ice, then separately homogenized in a sucrose buffer 0.25 M, sodium phosphate 50 mM, EDTA 10 mM and DTT 0.1 mM, (pH 7.4), in a glass homogenizer with a Teflon pestle. The homogenate was then centrifuged 10 min at 1300 *g* and 20 min at 10,000 *g*. The supernatant was decanted and recentrifuged at 105,000 *g* for 1 hr. The microsomal pellet was then re-suspended in a sodium pyrophosphate buffer 100 mM, EDTA 10 mM and DTT 0.1 mM, (pH 7.4), and recentrifuged at 105,000 *g* for 1 hr to wash the microsomal fractions in order to eliminate the artefactual interferences of haemoglobin in the spectral measurements. The microsomal pellet was then re-suspended in 1 ml of potassium phosphate buffer 100 mM, EDTA 10 mM, DTT 1 mM and glycerol 20% (v/v), (pH 7.4), for 1 g of liver weight. All handlings were carried out at 4°. Microsomal fractions were stored at −80° for enzymatical measurements.

Purification of cytochromes P-450. The microsomes obtained from PB and 3-MC-treated rats were used for the purification of cytochrome P-450b and cytochrome P-450c respectively. The purification was made until homogeneity according to the procedure used by Guengerich *et al.* [9]. The molecular weight of these two isozymes was estimated by SDS–PAGE electrophoretic method [10]. It was 52 kD for cytochrome P-450b and about 55 kD for cytochrome P-450c. The specific contents in cytochrome P-450b and c were 12.5 and 14.2 nmoles per mg protein respectively (Fig. 2).

Preparation of antisera. Antiserum against the purified enzyme was raised in rabbits. The animals were inoculated subcutaneously with 150 μg of purified cytochrome P-450 isozyme mixed with 0.5 ml of Freund's complete adjuvant. Similar booster injections but using Freund's incomplete adjuvant, were given at 3-weeks intervals; each rabbit was bled from the ear vein 8 days after the second booster challenge. The anti cytochrome P-450b and anti cytochrome P-450c IgG were purified using a protein-A. Sepharose CL 4B column (Pharmacia), after 50% ammonium sulfate precipitation of antiserum proteins according to the procedure indicated by Pharmacia.

Specificity of antisera. Antisera raised to purified

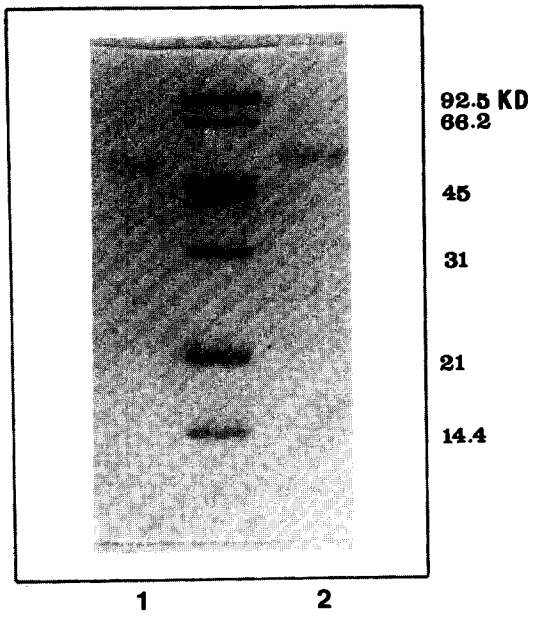

Fig. 2. Discontinuous SDS–PAGE: (1) purified cytochrome P-450b from PB treated rats microsomes; (2) purified cytochrome P-450c from 3-MC treated rats microsomes.

cytochrome P-450b and cytochrome P-450c in rabbits were examined for reactivity towards purified cytochrome P-450b and cytochrome P-450c, as well as towards other microsomal proteins, using double-immunodiffusion analysis.

Electrophoretic separation of proteins. Discontinuous SDS–PAGE was performed according to Laemmli [10]. The stacking and running gels were 5% and 10% w/v acrylamide respectively. Electrophoresis were performed at room temperature for 2 hr under a constant current of 25 mA.

Electroblotting. Protein transfers on nitrocellulose sheets (Schleicher and Schull, F.R.G.) were realized according to Burnette [11].

Spectral analysis. The binding of dantrolene to rat liver microsomes was examined by u.v.-visible difference spectra. Microsomes were diluted to 2 mg/ml of protein in 0.1 M potassium phosphate buffer (pH 7.4); dantrolene was dissolved in methanol. The difference spectra were recorded between 460–360 nm, after addition of increasing concentrations of dantrolene in the sample cuvette and of the same quantity on μl of methanol in the reference cuvette, using an Uvikon 820 (Kontron) spectrophotometer in a split beam mode. The system recommended by Jefcoate [12] with double compartment cuvettes was used for the titration of cytochrome P-450 with substrates that absorb in this region of the spectrum. All studies were conducted at room temperature. The relationship between absorbance and dantrolene concentration was analyzed using a double reciprocal plot representation for the determination of the apparent spectral dissociation constant K_s. The determination of the spin state of cytochrome P-450 was measured by n-octylamine titration according to ref. 12.

Enzyme assays. The protein content of the microsomal suspension was determined using the method of Lowry *et al.* [13] with bovine serum albumin as a standard. Cytochrome P-450 and cytochrome *b*5 were measured according to Omura and Sato [14]. Aminopyrine, ethylmorphine and benzphetamine *N*-demethylase activities were performed by the method of Yang and Strickhart at 5 mM final substrate concentration [15], paranitroanisole *O*-demethylase by the method of Netter and Seidel [16], 7-ethoxycoumarin *O*-deethylase, 7-ethoxyresorufin *O*-deethylase and aniline hydroxylase activities were measured according to Ullrich and Weber [17], Burke and Mayer [18] and Mazel [19] respectively. NADPH cytochrome *c* reductase was determined as described by Strobel and Digman [20].

UDP-glucuronosyltransferases were determined according to the Mulder and Van Doorn method [21] modified by Colin-Neiger *et al.* [22] at a final substrate concentration of 0.5 mM, with the following aglycones: 1-naphthol, 4-nitrophenol, 4-phenyl-7-hydroxycoumarin, nopol and chloramphenicol. UDP-glucuronic acid concentration was 4.5 mM. Bilirubin-UDP-glucuronosyltransferase was measured by the Heirwegh *et al.* method [23] with a final bilirubin concentration of 0.125 mM. Epoxide hydrolase was estimated with benzopyrene oxide by the method of Dansette *et al.* [24] and by immunoassays according to Zhiri *et al.* [25], using a non-competitive system with horse radish peroxidase labeled IgG against purified epoxide hydrolase from phenobarbital treated rat liver microsomes. Gamma-glutamyltransferase was measured by Szasz's method [26], and microsomal glutathion-*S*-transferase by the Habig and Jakoby method [27] with dinitrochlorobenzene as a substrate.

Statistical analysis. All direct comparisons to a single control group were conducted with the unpaired Students' *t*-test.

RESULTS

Binding of dantrolene to liver microsomes

We observed no binding of dantrolene to liver microsomes obtained from control or PB treated rats. A difference type I binding spectrum was obtained only with liver microsomes obtained from 3-MC treated rats. The pattern of spectral changes obtained was characterized by an absorption peak at 385–390 nm and a trough at 420 nm (Fig. 3). The spectral dissociation constant in this case was 5.5 μM, which indicated a very high affinity for the type I binding site, ΔA_{max} was 0.01. Liver microsomes from dantrolene treated rats showed only weak type I binding spectrum.

Cytochrome P-450-dependent monooxygenase activities (Table 1)

Very substantial decreases in the *N*-demethylations of aminopyrine, benzphetamine and ethylmorphine were observed in liver microsomes of dantrolene treated rats. Conversely, the *O*-demethylation of paranitroanisole and the *O*-deethylation of 7-ethoxycoumarin were increased by 10% and 40%, respectively. The *O*-deethylation of 7-ethoxyresorufin was increased fivefold, while in

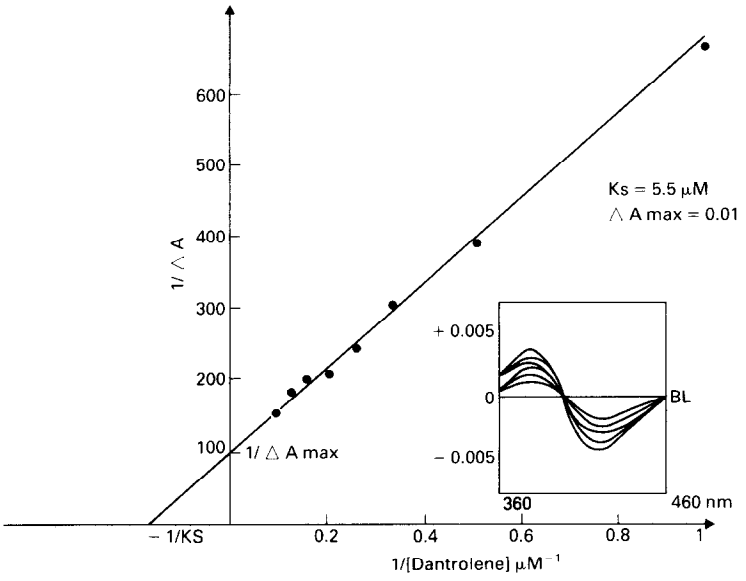

Fig. 3. Binding of dantrolene to liver microsomes from 3-MC treated rats. ΔA was the difference of the absorbance between 390 and 420 nm.

the same time, the cytochrome P-450 content was decreased by about 30%. No significant changes either in the NADPH cytochrome P-450 reductase activity, in the cytochrome *b5* content or in the aniline hydroxylase activity, were observed.

The spin state of microsomal cytochrome P-450 was also changed after dantrolene treatment: 40% of liver microsomal cytochrome P-450 was in the high spin state, while in liver microsomes from control animals, the high spin state was only 24%.

Effects of dantrolene pretreatment on other enzyme activities

The activity of UDP-glucuronosyltransferase (GT1) whose aglycones are 1-naphthol, p-nitrophenol and 4-phenyl-7-hydroxycoumarin was increased approximatively twofold, whereas UDP-glucuronosyltransferase (GT2) (nopol, chloramphenicol) and bilirubin UDP-glucuronosyltransferase remained unchanged (Table 2). Epoxide hydrolase estimated by enzyme activity determination and by immunoassays (ELISA) was increased threefold. The microsomal gamma-glutamyltransferase was increased by 20% (Table 3), whereas glutathion-*S*-transferase remained unchanged (data not shown).

Cytochrome P-450c induction in liver microsomes of dantrolene treated rats

The ability to identify individual isozymes of cytochrome P-450 by immunological reactivity provided an instrument to compare dantrolene with various agents (3-MC, PB) known to induce cytochrome P-450.

The electroblotting data presented in Fig. 4B indicate that anti-cytochrome P-450b antiserum recognized preferentially the microsomal cytochrome P-450 from PB treated rats. We observed a weak band in control, 3-MC and dantrolene treated rat microsomes with a molecular weight of about 51 kD. On the other hand, we observed a band with a higher

Table 1. Cytochrome P-450 dependent monooxygenase activities in rat liver microsomes after dantrolene treatment

Monooxygenase system	Control (N = 12)	Dantrolene treated rats (N = 12)
Cytochrome P-450 (nmole/mg prot)	0.87 ± 0.08	0.61 ± 0.08***
Cytochrome *b5* (nmole/mg prot)	0.32 ± 0.02	0.40 ± 0.03**
Cytochrome *c* reductase (nmole/min/mg prot)	144.5 ± 9.5	151.6 ± 14.6 (NS)
Aminopyrine *N*-demethylase (nmole/min/mg prot)	3.74 ± 0.29	1.94 ± 0.41***
Benzphetamine *N*-demethylase (nmole/min/mg prot)	3.99 ± 0.26	1.76 ± 0.34***
Ethylmorphine *N*-demethylase (nmole/min/mg prot)	4.31 ± 0.32	1.54 ± 0.22***
Paranitroanisole *O*-demethylase (nmole/min/mg prot)	0.60 ± 0.04	0.67 ± 0.08*
7-Ethoxycoumarin *O*-deethylase (nmole/min/mg prot)	0.28 ± 0.03	0.40 ± 0.06***
7-Ethoxyresorufin *O*-deethylase (pmole/min/mg prot)	22.0 ± 8.05	96.25 ± 19.6***
Aniline Hydroxylase (nmole/min/mg prot)	0.68 ± 0.08	0.64 ± 0.05 (NS)

N = number of animals; NS = non-significant.
*P ≤ 0.05 ** P ≤ 0.01 *** P ≤ 0.001.

Table 2. UDP-glucuronosyltransferase activities in rat liver microsomes after dantrolene treatment

UDP-glucuronosyltransferase (nmole/min/mg prot.)	Control (N = 6)	Dantrolene treated rats (N = 6)
GT1		
1-Naphthol	48.04 ± 4.35	78.90 ± 15.90***
4-Nitrophenol	37.49 ± 3.42	72.49 ± 15.00***
4-Phenyl-7-hydroxycoumarin	73.54 ± 6.33	123.57 ± 16.91***
GT2		
Nopol	22.06 ± 1.90	26.96 ± 2.15 (NS)
Chloramphenicol	3.60 ± 0.36	3.61 ± 0.3 (NS)
GT3		
Bilirubin	0.65 ± 0.09	0.66 ± 0.04 (NS)

N = number of animals; NS = non-significant.
* $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$.

Table 3. Rat liver microsomal gamma-glutamyltransferase (GGT) and epoxide hydrolase after dantrolene treatment

	GGT (nmole/min/mg prot.)	Epoxide hydrolase (nmole/min/mg prot.)	(μg/mg prot.)
Control (N = 6)	0.60 ± 0.02	7.76 ± 0.2	11.58 ± 5.06
Dantrolene treated rats (N = 6)	0.83 ± 0.1*	20.91 ± 0.2***	23.7 ± 3.7***

N = Number of animals.
* $P \leq 0.05$. *** $P \leq 0.001$.

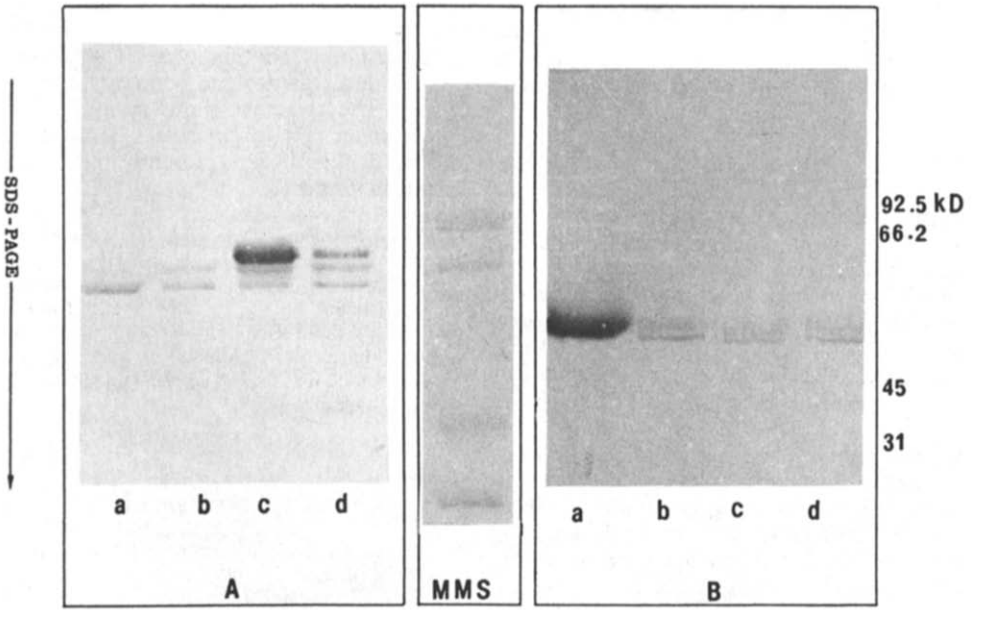

Fig. 4. Immunoblot analysis of microsomal hepatic rat cytochrome P-450 isozymes: (A) cytochrome P-450c antiserum stained bands, (B) cytochrome P-450b antiserum stained bands. a—microsomes from PB treated rats, b—control microsomes, c—microsomes from 3-MC treated rats, d—microsomes from dantrolene treated rat. MMS, molecular weight standards.

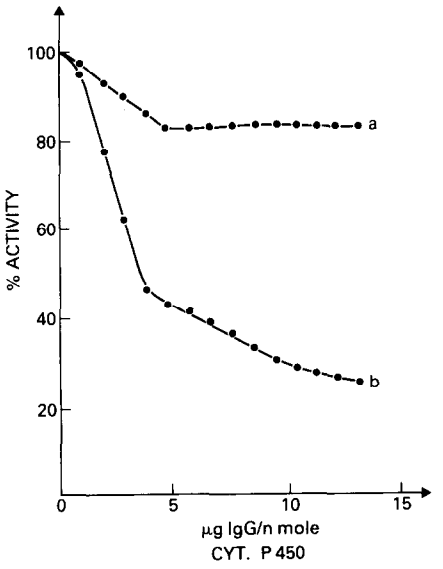

Fig. 5. Immunoinhibition of the activity 7-ethoxyresorufin O-deethylase by purified antiserum raised against purified cytochrome P-450c with (a) microsomes from control rats; (b) microsomes from dantrolene treated rats. Incubation was made over 16 hr at 4° in a tube containing 1 ml of microsomal protein at the concentration of 5 mg/ml.

molecular weight (55 kD), corresponding to microsomal cytochrome P-450c induced by 3-MC (Fig. 4A). This band was stained with anti-cytochrome P-450c antiserum. We observed a similar band in microsomes from dantrolene treated rats but this band was absent in microsomes from control and PB-treated rats. Figure 5 shows the immunoinhibition curve of 7-ethoxyresorufin *O*-deethylase activity with anti-cytochrome P-450c antiserum in dantrolene treated and control rat liver microsomes. An inhibition of about 80% of the activity was observed in the case of microsomes from dantrolene treated rats.

DISCUSSION

In the present work, we demonstrated by determination of cytochrome P450c-dependent monooxygenase activities, immunoelectrophoresis and by immunoinhibition that the cytochrome P-450c isozyme was not inhibited but induced after *in vivo* treatment with dantrolene. We also observed that the aniline hydroxylase activity which is induced by ethanol [28] isosafrole or arochlor [9], remained unchanged.

Dantrolene sodium has been described formerly as an inhibitor of some cytochrome P-450 dependent activities as well as a depleting agent of cytochrome P-450 content [29]. Other authors have described the inhibition of the PB-induced cytochrome P-450-dependent activities by dantrolene. Roy *et al.* [29] have observed that PB associated with dantrolene during treatment of rats decreased the inhibitory action of dantrolene on the hepatic monooxygenase activity, which tended to prove that the isozyme inhibited by dantrolene can be induced by PB.

Our data support the hypothesis that the inhibitory effect of dantrolene was selective of the cytochrome P-450 isozyme, inducible by PB, and that an opposite effect was obtained with the isozyme induced by 3-MC.

The induction of cytochrome P-450c has been described as a potential sign of a toxic mechanism, especially as cytochrome P-450c appears to be associated with malignancy and the production of reactive intermediates [7]. It remains questionable whether the induction of cytochrome P-450c is or is not an index for toxicity. It is worth pointing out that dantrolene liver toxicity was never reproduced in animal models, except a hypothesis concerning the hamster [6]. It is true that the hamster possesses high cytochrome P-450c-dependent monooxygenase activities [18] but also high production of mercaptans [6]. In humans, the liver cytochrome P-450c can vary by a factor of 1 to 6 [30] with environmental conditions. This casual variation might be related to the unexplained rate (0.3%) of acute toxic reactions caused by dantrolene. Our observation that dantrolene was exclusively bound to cytochrome P-450c induced by 3-MC, giving a type I substrate spectrum, tends to indicate that dantrolene is metabolized by cytochrome P-450c. Moreover, nitrofurantoine, a structural analogue of dantrolene, was also reported to be more actively metabolized after treatment of rats with 3-MC [31]. Comments concerning the elevation of epoxide hydrolase [32], and UDP-glucuronosyltransferase (GT1) [33] also indicate that these activities could constitute an index for the cell toxicity of a drug, which could be mediated by toxic intermediates (epoxides).

The present work allowed testing of the influence of dantrolene on the cytochrome P-450-dependent oxidative pathway and on the phase II drug metabolizing enzymes, sequentially involved. Our data show that epoxide hydrolase and gamma-glutamyltransferase activities were also slightly increased.

Nevertheless, another hypothesis concerning liver toxicity after dantrolene administration has to be tested. The reductive pathway may lead to toxic intermediates [34, 35] in aerobic and anaerobic conditions. This pathway is presently under investigation in this laboratory.

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