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ISOLATION OF A HIGH SPIN FORM OF CYTOCHROME P-450 INDUCED IN RAT LIVER BY 3-METHYLCHOLANTHRENE

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SUMMARY: A form of cytochrome P-450 (P-450 MC_1) has been isolated from the livers of 3-methylcholanthrene-treated rats. The molecular weight is 54,500 and the heme iron is in the high spin configuration which clearly differenciates this form from the other major cytochrome induced by 3-methylcholanthrene (P-450 MC_2). Whilst MC_2 actively dealkylated 7-ethoxycoumarin and 7-ethoxyresorufin, MC_1 was only active with 7-ethoxyresorufin. Ouchterlony immunodiffusion analysis and ELISA showed that anti MC_1 and anti MC_2 reacted with both MC_1 and MC_2 but preferentially with the homologous antigen. Both anti MC_1 and anti MC_2 cross-reacted strongly with microsomes from 3-methylcholanthrene, Aroclor 1254 and isosafrole-treated rats and also, but much weaker, with microsomes from phenobarbital, trans-stilbene oxide and chlofibrate-treated as well as untreated rats. Both MC_1 and MC_2 are induced by the same inducers, 3-methylcholanthrene, Aroclor 1254 and also isosafrole, whilst phenobarbital, trans-stilbene oxide and chlofibrate did not induce either of them, which shows that MC_1 and MC_2 are under similar control by various types of inducers, but MC_1 was present in control microsomes at higher levels than MC_2.

INTRODUCTION: The induction of different forms of cytochrome P-450 with clearly distinguishable structures and substrate specificities represents an important factor in the balance between the detoxification and toxicity of foreign compounds. For example it is well known that the generation of ultimate carcinogenic metabolites of several polycyclic hydrocarbons is increased significantly by treatment of the animals with compounds such as 3-methylcholanthrene (1).

In untreated animals it appears that there are many forms of cytochrome P-450 (2,3). To date nearly all inducible enzymes have been shown to be present in control microsomes (3,4), but usually at very low concentrations (3). The number of endogenous forms induced by treatment with inducing agents is still undetermined, but this information and the relative proportions of the

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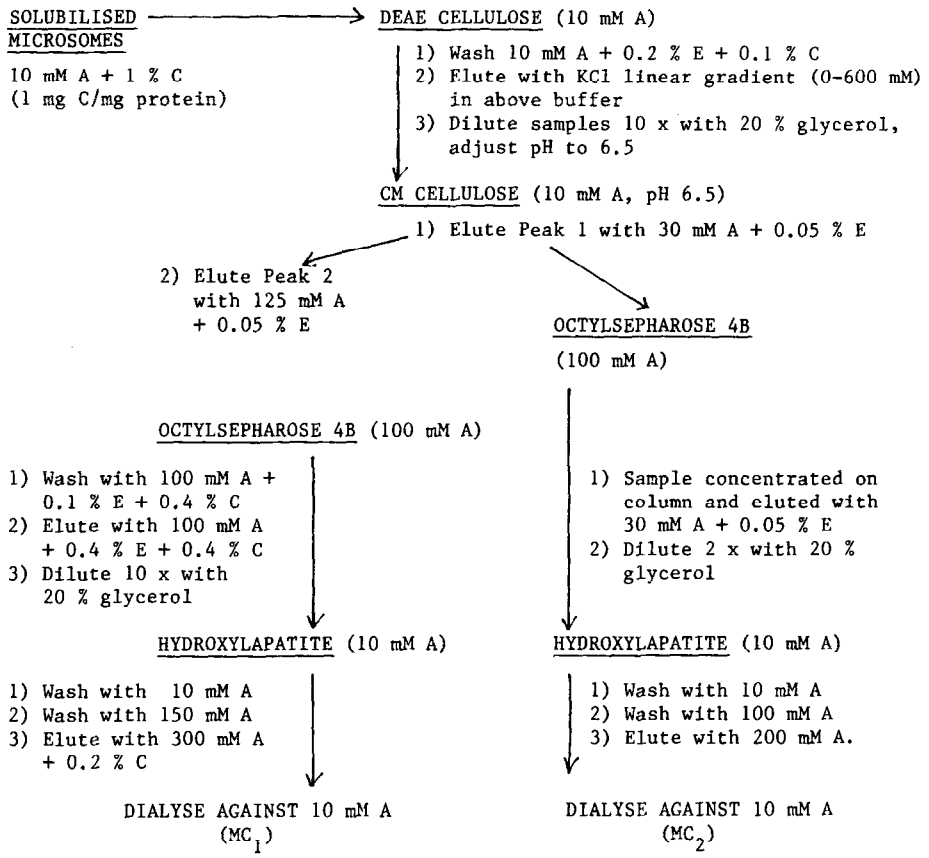
proteins induced is necessary to understand function and regulation of cytochrome P-450. In this paper we describe the isolation and characterisation of a form of P-450 induced by 3-methylcholanthrene, that is clearly distinguishable from the well characterised cytochrome induced by this compound (3). This form is also induced by Aroclor 1254 and isosafrole.

MATERIALS AND METHODS:

Male Sprague Dawley rats (200 g) treated with 3-methylcholanthrene, (20 mg/kg body weight, i.p. in sun flower oil 96, 48 and 24 hours before sacrifice) were used for the enzyme isolations. The dosage regimes for other inducing agents were: Aroclor 1254, 500 mg/kg, i.p. in corn oil, 1 week before use; chlofibrate and trans-stilbene oxide, 400 mg/kg i.p. in corn oil, isosafrole, 150 mg/kg, i.p. in corn oil, 96, 48 and 24 h before use; and phenobarbital, 80 mg/kg i.p. in 0.9 % NaCl, 96, 48 and 24 h before use. Liver microsomes were prepared according to previously published procedures (5). The cytochrome purification was carried out using modified procedures of those described by Wolf et al. (5,6) and Ryan et al. (7). A flow diagram for the procedure is shown below. Two cytochrome fractions MC_1 and MC_2 were obtained using this procedure. The specific content of these samples were 16.5 and 16.4 nmol/mg protein and the yields were 0.5 and 4.0 % of the original cytochrome content for MC_1 and MC_2, respectively. Cytochrome P-450 reductase was separated from the other monooxygenase components (5) and purified by affinity chromatography by the method of Yasukochi and Masters (8).
Antibodies to the two proteins were raised in New Zealand white rabbits (200 µg in 1 ml Freunds complete adjuvant s.c. followed by 3 further administrations of 100 µg in 1 ml incomplete adjuvant at weekly intervals). Double immunodiffusion was carried out using the method of Ouchterlony (9). Enzyme-linked immunosorbent assays (ELISA) were carried out according to the method of Burger et al. (10) using a peroxidase conjugated antibody. Absorbance values were read at 480 nm on a Titer Tek Multiscan (Flow Labs). SDS slab gel electrophoresis was according to the method of Laemmli (11). Cytochrome P-450 was determined by the method of Omura and Sato (12), protein by the method of Lowry et al. (13). The conditions used for the reconstitution of monooxygenase activity have been reported previously (14) with the exception that synthetic dilauroylphosphatidylcholine was used. The direct fluorimetric assay for 7-ethoxycoumarin and 7-ethoxyresorufin was used (15). All chemicals were of high purity and were obtained from commercial sources.

RESULTS

The absolute spectra of the two cytochrome fractions obtained using the procedure described above are shown in Figure 1. Form MC_1 is high spin in the native state with characteristic absorption peaks at 395 and 648 nm. The other cytochrome (MC_2) is low spin with a Soret peak at 419 nm. The ferrous cytochrome- carbon monoxide complex of both cytochromes had maxima at 447 nm. The monomeric molecular weights of the two proteins were significantly different being 54,500 and 57,000 for MC_1 and MC_2, respectively (Figs. 2a and 2b). In Ouchterlony double immunodiffusion analysis an antibody to MC_1, produced in



Procedure for the purification of P-450 forms from 3-methylcholanthrene-treated rats. A = phosphate buffer containing 20 % glycerol, 0.1 mM dithiothreitol and 0.1 mM EDTA. The pH was 7.7 unless otherwise stated. E = Emulgen 911. C = sodium cholate. ( ) = Equilibration buffer.

Flow diagram.

rabbits, cross-reacted very weakly with MC_2. Anti MC_2 did not appear to cross-react with MC_1 (Fig. 3a). Using microsomal preparations from animals treated with reagents which induce cytochrome P-450 lines of identity were observed using either antibody and microsomes from 3-methylcholanthrene or Aroclor 1254 or isosafrole-treated animals (Fig. 3b). The cross-reactivity of anti MC_1 and anti MC_2 was strong with microsomes from 3-methylcholanthrene, Aroclor 1254 and isosafrole-treated rats but with microsomes from chlofibrate, phenobarbital and trans-stilbene oxide-treated as well as untreated rats much weaker or nil: precipitation lines were only visible with anti MC_1 versus microsomes from trans-stilbene oxide-treated and untreated rats. Precipitin lines from weakly cross-reacting antigens in microsomes are difficult to clearly visualize by

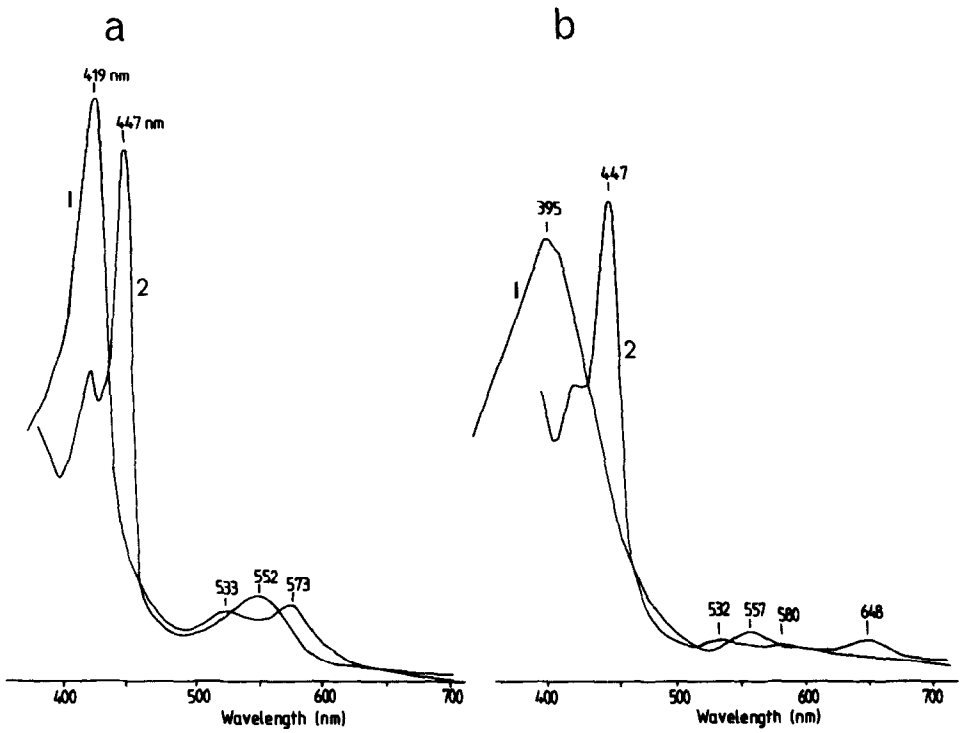


Figure 1. Spectra of cytochromes MC_1 (b) and MC_2 (a) isolated from 3-methylcholanthrene-treated rats. 1 = Ferric cytochrome, 2 = Ferrous cytochrome-CO complex.

Ouchterlony double diffusion analysis since low concentrations of microsomes may not result in visible precipitin lines and high concentrations of microsomes may overshadow weak precipitin lines. Therefore, possible structural analogy between MC_1 and MC_2 was further investigated using an ELISA (Table 1). Anti MC_1 and anti MC_2 cross-reacted with their analogous antigens. In addition anti MC_1 and anti MC_2 cross-reacted weakly but clearly also with MC_2 and MC_1, respectively. Anti MC_1 and anti MC_2 cross-reacted strongly with microsomes from 3-methylcholanthrene, Aroclor 1254 and isosafrole treated animals and also but much weaker with microsomes from control, phenobarbital, trans-stilbene oxide or chlofibrate-treated animals. The higher absorption units compared to control microsomes in the ELISA show that MC_1 and MC_2 or proteins with common antigenicity are induced by 3-methylcholanthrene, Aroclor 1254 and also by isosafrole, but not by phenobarbital, trans-stilbene oxide and chlofibrate, which shows that they are under similar control by various types of

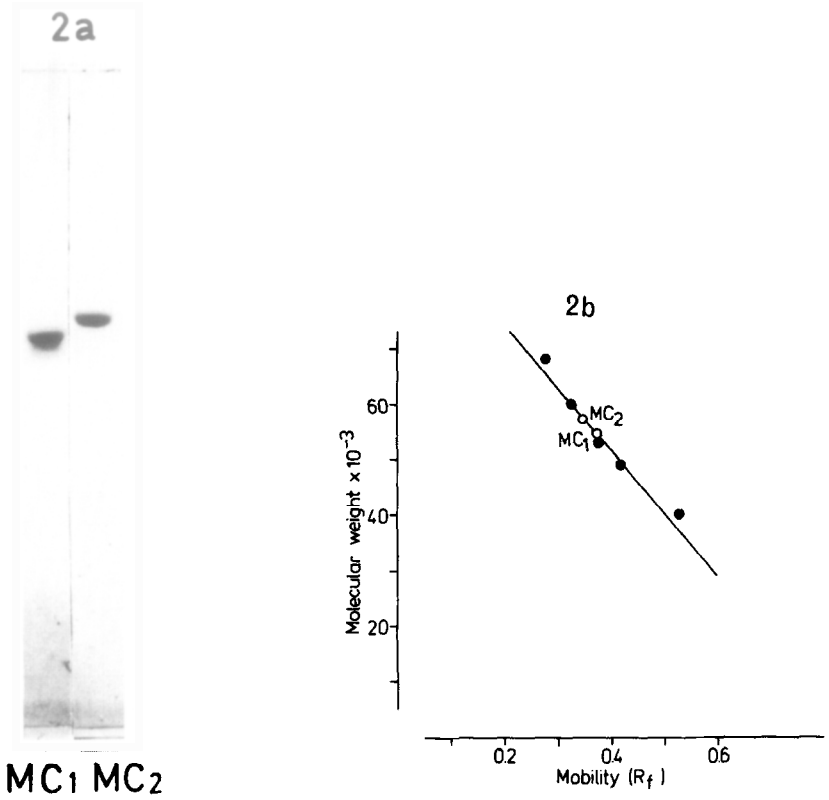


Figure 2. a = SDS-polyacrylamide gel electrophoresis of MC_1 (5 µg) and MC_2 (5 µg); b = molecular weight determination of MC_1 and MC_2. The standards used were bovine serum albumin (67.000), catalase (60.000), glutamic dehydrogenase (53.000), fumarase (49.000), aldolase (40.000).

inducers. Table 1 shows also that MC_1 or proteins with common antigenicity are present in microsomes from untreated rats at higher levels then MC_2.

Both MC_1 and MC_2 were active in the metabolism of 7-ethoxyresorufin with turnover numbers of 10.1 and 70.3 respectively. With 7-ethoxycoumarin only MC_2 was active with a turnover number of 9.0, whilst no reaction was detectable with MC_1 (turnover number < 0.1).

DISCUSSION

Two clearly distinguishable forms of cytochrome P-450 have been isolated from the livers of 3-methylcholanthrene-treated rats. Both of these forms are induced by Arochlor 1254 and isosafrole as well as 3-methylcholanthrene. Form MC_2 appears to be the same as the well characterised form reported by Ryan et al. as form c (7). Form MC_1 has not previously been reported in microsomes

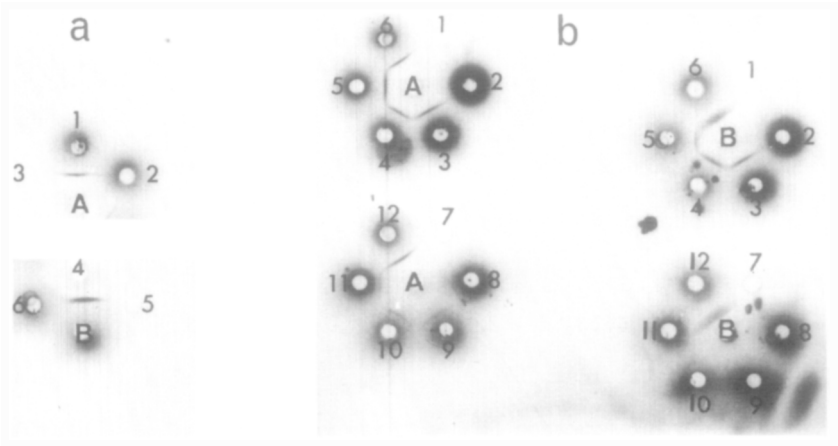


Figure 3. Immunodiffusion analysis of anti MC_1 and anti MC_2 against purified P-450's and liver microsomes from animals treated with different inducing agents. Wells A and B contained rabbit IgG (50 µg) containing anti MC_1 and anti MC_2, respectively. a. Wells contained: 1, P-450 MC_1; 2, P-450 MC_2; 3, buffer; 4, P-450 MC_2; 5, P-450 MC_1; 6, Buffer. Protein concentration 10 µg/well. b. Wells 1-12 contained the following microsomal preparations: 1, buffer; 2, Chlofibrate; 3, Aroclor 1254; 4, 3-methylcholanthrene; 5, isosafrole; 6, homologous antigen; 7, buffer; 8, phenobarbital; 9, trans-stilbene oxide; 10, control; 11, control; 12, homologous antigen. The microsomes were solubilised with cholate (1 mg/mg protein). The wells contained 40 µg protein. The agarose gels contained 0.2 % Emulgen 911. This experiment was repeated three times with the same results.

from 3-methylcholanthrene-treated animals. The isolated cytochrome is high spin. A high spin cytochrome was first isolated from liver microsomes from 3-methylcholanthrene or ß-naphthoflavone-treated rabbits (16,17). Since then high spin enzymes have been isolated from microsomal fractions from rats treated with isosafrole (18) or 3,4,5,3',4',5'-hexachlorobiphenyl (HCB, 19), a component of Arochlor 1254. The data presented here strongly indicates that

Table 1. ELISA for the detection of MC_1 and MC_2 in liver microsomal samples from rats treated with various inducing agents.

	P-450 MC_1	P-450 MC_2	Absorption (units) MC	ISO	ARO	PB	TSO	CHLO	CONT.
Anti MC_1	1.02	0.23	1.05	1.33	1.13	0.5	0.47	0.16	0.45
Anti MC_2	0.12	1.32	1.07	1.15	1.05	0.17	0.11	0.09	0.18

20 µl of IgG (0.01 mg/ml) and 10 µl of P-450 (10 µg/ml) and microsomal (0.4 mg/ml) samples were used per well. Microsomal samples (0.4 mg/ml) were solubilized with sodium cholate (1.6 mg/ml). The animals were treated with MC, methylcholanthrene; ISO, isosafrole; ARO, Aroclor 1254; PB, phenobarbital; TSO, trans-stilbene oxide and CHLO, chlofibrate. CONT. = control microsomes. This experiment was repeated and values did not vary more than 15 % from those shown above.

3-methylcholanthrene induces a protein (MC_1), which is the same as that induced by isosafrole or HCB. The induction of a high spin and low spin cytochrome by one reagent is not unique to rats. Johnson and Muller-Eberhard (20) have made similar observations in rabbits using 2,3,7,8-tetrachlorodibenzo p-dioxin as inducing agent. Whether isosafrole induces just the high spin enzyme in this species has not been determined. It would appear that many cytochrome forms are induced concomitantly by inducing agents. Vlasuk et al. reported 3 phenobarbital-inducible cytochrome P-450's in some rat colonies (21). Evidence for the induction of two cytochromes by 3-methylcholanthrene-type inducers, based on the appearence of protein bands in the 50-60,000 molecular weight region has been reported (22,23). The two major protein bands probably represent the cytochromes isolated here. Lau and Strobel (24) isolated 5 cytochrome forms from rats treated with ß-naphthoflavone. Unfortunately it was not demonstrated whether these represented inducible enzymes, or whether one of them was high spin. The form described as form 4 has a similar molecular weight and carbon monoxide spectrum to MC_1.

Since the data presented in this report indicate that MC_1 is present in microsomes from 3-methylcholanthrene-treated rats at similar levels and in control microsomes even at higher levels than MC_2, the potential role of MC_1 in activation of chemical carcinogens should be investigated.

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