Arrhenius plots of temperature dependence of ATPase activity of SR membranes in HChE and of proteoliposomes reconstituted from these membranes are illustrated in Fig. 3. It will be clear from Fig. 3 that replacement of the native lipid environment of Ca-ATPase by OL did not restore the normal character of the graph of temperatre dependence of activity, i.e., did not shift the kink into the low-temperature region (20°C), as is characteristic of proteoliposomes and SR membranes of control animals [3].

The results thus show that modification of the temperature dependence of Ca-ATPase activity in HChE is connected with changes in the polypeptide which performs the catalytic function and is not the result of a change in the phospholipid environment of the enzyme.

## LITERATURE CITED


1. I. L. Kuz'mina, A. A. Timofeev, S. B. Germanov, et al., Byull. Eksp. Biol. Med., No. 2, 163 (1985).
2. V. B. Ritov, Biokhimiya, 36, 393 (1971).
3. V. B. Ritov and M. K. Murzakhmetova, Biokhimiya, 48, No. 3, 415 (1983).
4. L. V. Stoida, Byull. Eksp. Biol. Med., No. 10, 38 (1983).
5. L. V. Stoida and A. A. Boldyrev, Byull. Eksp. Biol. Med., No. 7, 32 (1978).
6. A. A. Timofeev, I. L. Kuz'mina, O. A. Azizova, and G. V. Chernysheva, Byull. Eksp. Biol. Med., No. 1, 55 (1985).
7. G. V. Chernysheva, L. V. Stoida, and I. L. Kuz'mina, Byull. Eksp. Biol. Med., No. 3, 293 (1980).
8. L. R. Jones, S. W. Maddock, and H. R. Besch, J. Biol. Chem., 255, 9971 (1980).


# MONOCLONAL ANTIBODY STUDY OF CYTOCHROME P-450 ISOFORMS IN THE LIVER OF RATS TREATED WITH PHENOBARBITAL, 3-METHYLCHOLANTHRENE, AND AROCLOR 1254


A. Yu. Kolyada



UDC 612.351.11:577.152.112].014.46:615.214.24:547.854.5

KEY WORDS: cytochrome P-450; monoclonal antibodies; liver microsomes.


Cytochrome P-450, the terminal stage in the enzyme system oxidizing various exonobiotics (including drugs, carcinogens, and so on), is found in the body as a family of isoforms. As a result of introduction of various inducing agents into the body, different isoforms of cytochrome P-450 are synthesized. The distribution of these isoforms in the tissues of the body varies [12], and the isoforms themselves differ from each other in different tissues [10]. The distribution of these isoforms among the cells of an organ also is not uniform [2].

This paper describes an attempt to discover, with the aid of three types of monoclonal antibodies, the distribution of certain isoforms of cytochrome P-450 in the liver of rats treated with various inducers.

## EXPERIMENTAL METHOD

BaLB/c mice were immunized with cytochrome P-450 isolated from the liver of male Wistar rats. The rats were treated beforehand with phenobarbital in a dose of 70 mg/kg body weight or 3-methylcholanthrene in a dose of 40 mg/kg body weight. Cytochrome P-450 was isolated by the method in [7]; the procedure stopped at the first stage of purification (i.e., at precipitation with ammonium sulfate). Spleen cells of immune mice were fused with myeloma cells of strain P3-X-63 Ag 8.653 by the standard method [3]. The resulting clones were analyzed for the presence of staining on sections of rat liver by the immunofluorescence method. Paraffin


Department for the Study of Carcinogenic Agents, Research Institute of Carcinogenesis, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Trapeznikov.)


Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 4, pp. 422-425, April, 1986. Original article submitted June 3, 1985.

0007-4888/86/0004-0445$12.50

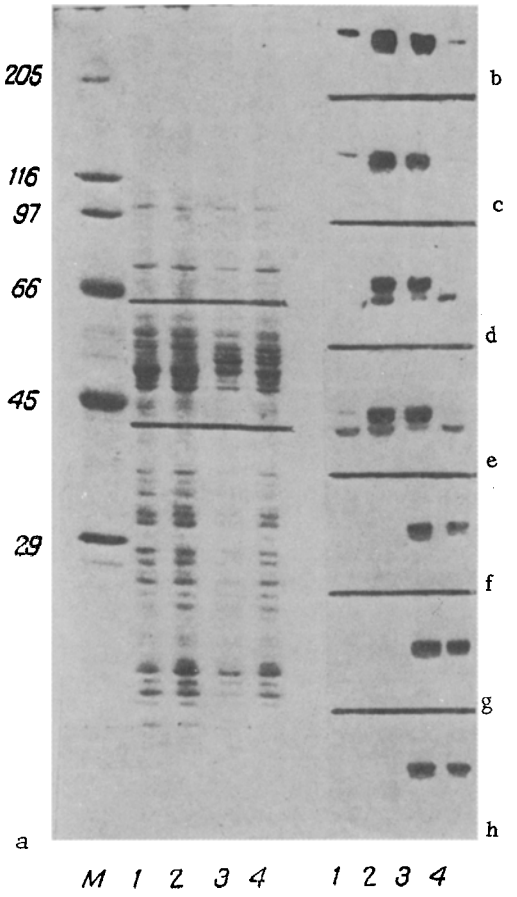


Fig. 1. Immunoblotting study of microsomal preparations stained with monoclonal antibodies to cytochrome P-450. 1) Liver microsomes from intact rat; 2) liver microsomes from rat treated with phenobarbital; 3) liver microsomes from rat treated with aroclor 1254; 4) liver microsomes from rat treated with 3-methylcholanthrene. M) Markers of molecular weights. a) Gel strained with Coomassie G-250; b) filter stained with CP-6A7 antibodies, c) filter stained with CP-2D5 antibodies, d) with CP-2F2 antibodies, e) with CP-3A2 antibodies, f) with CP-11E4 antibodies, g) with CP-12C10 antibodies, h) with CP-13A10 antibodies. Filters stained by the two-layer method: monoclonal mouse antibodies + rabbit antibodies to mouse γ-globulins, conjugated with peroxidase. Black stripes on gel (a) stained with Coomassie G-250 indicate part of gel an impression of which is contained in each of the seven filters stained with monoclonal antibodies (B-K). Numbers on left show molecular weights (in kilodaltons).

sections were stained by the method described previously [1]. To test clones obtained from mice immunized with cytochrome P-450 from the liver of rats treated beforehand with phenobarbital, sections also from the liver of rats treated with phenobarbital were used. To test clones obtained from mice immunized with cytochrome P-450 from the liver of rats treated beforehand with 3-methylcholanthrene, sections from the liver of rats also treated with 3-methylcholanthrene were used. Clones whose antibodies did not change the nuclei of the hepatocytes, but stained their cytoplasm intensely, and which also stained hepatocytes located near the central veins more strongly than those near the portal veins, were selected. The selected clones were recloned twice. For electrophoresis in a polyacrylamide gradient gel (7-15%) in the presence of sodium dodecylsulfate, the system described in [5] was used. Immunoblots [14] were stained by antibodies against mouse γ-globulins, conjugated with peroxidase.

To obtain preparations of microsomes and liver sections Wistar rats were used. The following substances were injected into the rats: phenobarbital (from Serva, West Germany) in a single dose of 80 mg/kg 1 day before sacrifice, 3-methylcholanthrene (from Fluka, Switzerland) in a single dose of 20 mg/kg 2 days before sacrifice, aroclor 1254 (from Analabs) in a single dose of 500 mg/kg 4 days before sacrifice, pregnenalone-carbonitrile (from Searle, England) in a dose of 80 mg/kg daily for 3 days before sacrifice, imidazole (from Sigma, USA) in a dose of 200 mg/kg daily for 3 days before sacrifice, and β-naphthoflavone (from Sigma)

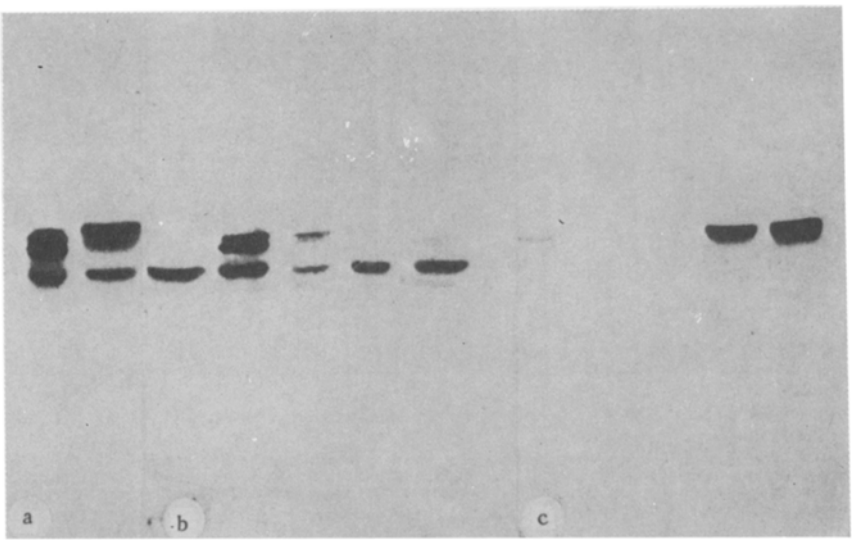


Fig. 2. Immunoblotting study of microsomal preparations stained with monoclonal antibodies to cytochrome P-450. 1) Liver microsomes from rats treated with imidazole, 2) with phenobarbital, 3) with pregnenalone-carbonitrile (PCN), 4) with β-naphthoflavone, 5) with 3-methylcholanthrene. a) Filter stained with antibodies CP-6A7 + CP-11E4; b) filter stained with CP-2F2, c) filter stained with CP-11E4.

in a dose of 80 mg/kg daily for 3 days before sacrifice. All substances were injected intraperitoneally: phenobarbital and imidazole in physiological saline, the rest in sunflower oil. Cytochrome P-450 was detected in the sections by the indirect immunofluorescence method using antibodies against mouse γ-globulins, labeled with fluorescein isothiocyanate.

## EXPERIMENTAL RESULTS

Altogether nine clones reacting in the immunoblotting method were obtained. Seven of these nine clones are described in this paper. Four clones, obtained by fusion of the spleen from a mouse immunized with cytochrome P-450 from a rat treated with phenobarbital (CP-6A7, CP-2D5, CP-2F2, and CP-3A2), reacted in the immunoblotting method as follows (Fig. 1).

Antibodies of the first two of these clones evidently reacted with two proteins with mol. wt. of about 50 kilodaltons (kD) in microsomes of rats induced with phenobarbital or aroclor 1254. These antibodies reacted weakly with microsomes from the liver of rats treated with 3-methylcholanthrene, or from intact animals. Antibodies of the second group (CP-2F2 and CP-3A2) also stained these two bands, and in addition, one other band of a protein with lower molecular weight, which was contained in all the antigens used (Fig. 1).

Three other positive clones were obtained on fusion of the spleen of a mouse immunized with cytochrome P-450 of a rat treated with 3-methylcholanthrene (CP-11E4, CP-12C10, and CP-13A10). They all stained two bands also; mol. wt. of the lighter of them, moreover, coincided with mol. wt. of the heaviest of the bands stained by the previous four clones. The second band stained by these three clones belonged to a protein with mol. wt. a little higher still (see Figs. 1 and 2). These two bands appeared only in microsomes of rats treated with 3-methylcholanthrene or with aroclor 1254. Even when tracks from microsomes of normal rats, overloaded with protein, were used for immunohistochemical staining, these bands could not be detected (data not given).

Comparison with data in the literature [4-9] indicates that the first two clones (CP-6A7 and CP-2D5) evidently react with an antigenic determinant common for isoforms P-450$_b$ and P-450PB/PCN-E (mol. wt. 50 and 52 kD, respectively), and the two next clones (CP-2F2 and CP-3A2) with P-450$_a$, P-450$_b$, and P-450PB/PCN-E (mol. wt. 48.5, 50, and 52 kD, respectively). The last three clones (CP-11E4, CP-12C10, and CP-13A10) react with P-450$_c$ and P-450$_d$ (mol. wt. 54 and 51 kD). Values of mol. wt. are taken from [4]. The antibodies of the last group which we obtained are evidently analogous to three clones obtained by Thomas et al. [13], which cross-

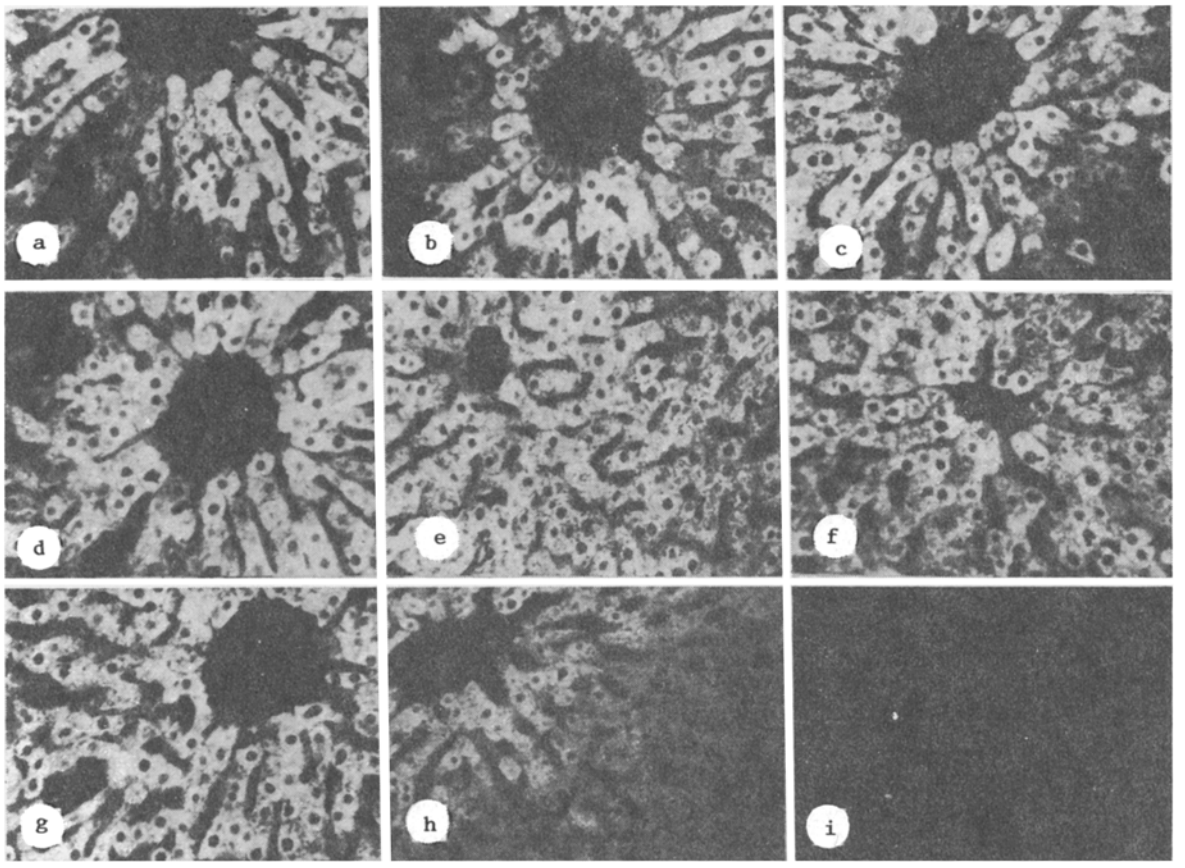


Fig. 3. Staining of liver sections with monoclonal antibodies to cytochrome P-450 by the indirect immunofluorescence method. a-h) Liver sections from rat receiving aroclor 1254; i) liver section from intact rat. a) Staining with antibodies of clone CP-6A7, b) CP-2D5, c) CP-2F2, d, i) CP-3A2, e) CP-11E4, f) CP-12C10, g) P-13A10, h) control, culture medium from myeloma X-63. 300 ×.

react with $P\text{-}450_c$ and $P\text{-}450_d$. By using additional antigens, such as liver microsomes from rats induced with pregnenalone-carbonitrile (PCN), it was in fact possible to obtain a band staining with antibodies CP-6A7, CP-2D5, CP-2F2, and CP-3A2, and equal in molecular weight to the heaviest band in the microsomes of rats treated with phenobarbital (Fig. 2). By using microsomes from rats treated with β-naphthoflavone, it was possible to obtain the same bands on immunoblotting as when microsomes from rats treated with 3-methylcholanthrene were used (Fig. 2). The use of microsomes from rats treated with imidazole showed that the antibodies obtained do not react with isoforms induced by this substance. The isoform which we interpreted as P-450PB/PCN-E, according to our data, has two common antigenic determinants with $P\text{-}450_b$ and one with $P\text{-}450_a$, which is in poor agreement with data indicating that the mRNA of this isoform does not hybridize with genes of P-450 isoforms of the phenobarbital family [11].

The results of immunohistochemical staining of liver sections from a rat treated with aroclor 1254, with the aid of the antibodies which we obtained, are given in Fig. 3. All these antibodies stained hepatocytes near the central veins more strongly than those near the portal veins. Incidentally, the gradient of staining appeared much brighter for isoforms induced with phenobarbital than for isoforms induced by 3-methylcholanthrene. Staining was observed in the liver of intact rats on account of interaction of CP-2F2 or CP-3A2 antibodies with $P\text{-}450_a$ (Fig. 1). Cells near the central veins were stained (Fig. 3).

## LITERATURE CITED

1. A. Yu. Kolyada, Byull. Eksp. Biol. Med., No. 7, 115 (1981).
2. J. Baron, J. Q. Redick, and F. P. Guengerich, Life Sci., 23, 2627 (1978).
3. G. Galfre, S. C. Howe, C. Milstein, et al., Nature, 266, 550 (1977).
4. F. P. Guengerich, G. A. Dannan, S. T. Wright, et al., Biochemistry (Washington), 21, 6019 (1982).

5. U. K. Laemmli, Nature, 227, 680 (1970).
6. A. Y. H. Lu and W. Levin, Biochem. Biophys. Res. Commun., 46, 1334 (1972).
7. D. E. Ryan, P. E. Thomas, D. Korzeniovski, et al., J. Biol. Chem., 254, 1365 (1979).
8. D. E. Ryan, P. E. Thomas, and W. Levin, J. Biol. Chem., 255, 7941 (1980).
9. D. E. Ryan, S. Jida, A. W. Wood, et al., J. Biol. Chem., 259, 1239 (1984).
10. C. J. Serabjit-Singh, C. R. Wolf, and R. M. Philpot, J. Biol. Chem., 254, 9901 (1979).
11. D. J. Simmons, P. A. Lalley, and C. B. Kasper, J. Biol. Chem., 260, 515 (1985).
12. B. J. Song, T. Fujino, S. S. Park, et al., J. Biol. Chem., 259, 1395 (1984).
13. P. E. Thomas, L. M. Reik, D. E. Ryan, et al., J. Biol. Chem., 259, 3890 (1984).
14. H. Towbin, T. Staehelin, and J. Gordon, Proc. Natl. Acad. Sci. USA, 76, 4350 (1979).

# STATE OF THE HEPATIC CYTOCHROME P-450 SYSTEM IN RATS VARIOUSLY PREDISPOSED TO EXPERIMENTAL ALCOHOLISM

T. G. Khlopushina, A. B. Kampov-Polevoi, E. M. Lysenkova, O. P. Kashevskaya, I. E. Kovalev, and Yu. V. Burov

UDC 616.89-008.441.13-056-092.9-07: 616.36-008.931:577.152.112]-074

KEY WORDS: experimental alcoholism; mono-oxygenase system; chronic alcoholization.

Chronic administration of ethanol to experimental animals is known to increase the intensity of metabolism not only of ethanol itself, but also of other xenobiotics of the microsomal fraction of the liver, in these animals [4]. However, information in the literature does not answer the question whether these changes in metabolism are the result of differences in the initial reactivity of the liver microsomes or whether they are secondary in character and are caused by chronic alcoholization.

It was accordingly decided to study activity of the cytochrome P-450-dependent mono-oxygenase system of the liver in animals selected on the basis of the strength of their initial alcohol motivation without contact with ethanol, and also the dynamics of cytochrome P-450 activity during voluntary alcoholization for 10 days.

## EXPERIMENTAL METHOD

Experiments were carried out on 44 noninbred male rats weighing 250-300 g. Predisposition to the development of experimental alcoholism was estimated by measuring the total time of immobilization (TTI) in a compulsory swimming test, by the method suggested by the writers previously [2]. Animals with a TTI of under 140 sec were classified as not predisposed to develop experimental alcoholism, those with a TTI of over 300 sec as animals predisposed to develop this experimental pathology. Thus, for the experiments 22 rats predisposed (TTI 308.3 ± 5.4 sec) and 22 rats not predisposed (TTI 130.0 ± 10.8 sec) to develop experimental alcoholism were selected for the experiments. Activity of the mono-oxygenase system of the liver was studied in some animals (11 from each group) after contact for 10 days with alcohol in individual cages measuring 40 × 12 × 15 cm, equipped with graduated bowls containing water and 15% ethanol solution. Biochemical investigations on the remaining animals were carried out after they had been kept under similar conditions for 10 days, but without contact with alcohol. The animals received food ad lib.

The state of the cytochrome P-450-dependent mono-oxygenase system of the liver was determined as follows. The animals were killed 24 h after withdrawal of ethanol. The microsomal fraction was obtained from liver homogenate of the experimental animals by differential centrifugation [5]. The protein concentration of the microsomes was determined by a modified Lowry's method [6]. The state of the hydroxylating complex was assessed from the content of

Laboratory of Immunopharmacology, Research Institute for Biological Testing of Chemical Compounds, Ministry of the Medical Industry of the USSR, Kupavna. Department of Neuropharmacology, Research Institute of Pharmacology, Academy of Medical Sciences of the USSR, Moscow.
(Presented by Academician of the Academy of Medical Sciences of the USSR A. V. Val'dman.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 4, pp. 425-426, April, 1986. Original article submitted April 4, 1985.

0007-4888/86/0004-0449$12.50  