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CHANGE IN THE MIXED FUNCTION OXIDASE SYSTEM OF LIVER MICROSOMES IN RATS TREATED WITH 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE[1]

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SUMMARY The mixed function oxidase systems of plasma membranes, the Golgi apparatus, and smooth and rough endoplasmic reticula of the livers of rats fed on a standard diet containing 0.06%(w/w) 3'-methyl-4-dimethylaminoazobenzene were investigated. The components and activities of the mixed function oxidase systems of the smooth endoplasmic reticulum and Golgi apparatus were especially reduced by the carcinogen. The activities for hydroxylation of anilines and demethylation of *p*-nitroanisole and the contents of cytochromes P-450 and b_5 of the submicrosomal fractions of the rats decreased considerably more than the activities of NADH- and NADPH-ferricyanide reductases. More than 90% of the ferri-cytochrome P-450 content of the 3'-methyl-4-dimethylaminoazobenzene induced microsomes at 20 K was in the low spin form.

INTRODUCTION

Administration of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) to rats for 6 months is known to induce hepatoma (Odashima, 1962) and to affect the cytochrome contents and activities of mixed function oxidase systems of whole liver microsomes (Nebert and Mason, 1963). However, the effects of 3'-Me-DAB on the amount and activity of each component of cytochrome P-450-linked mixed function oxidase systems of the Golgi apparatus, and smooth and rough microsomes, prepared from whole microsomes of rat livers, and on the spin forms of heme-irons in ferri-cytochromes P-450 and b_5 are still unknown.

Accordingly we studied the effects of oral and parenteral 3'-Me-DAB administration to rats on the activities of mixed function oxidase systems and the contents of spin forms of cytochromes in submicrosomal fractions of their livers.

1 A preliminary report of this work was presented at the 21st Annual Meeting of the Japanese Biochemical Society, Wakayama, June 1, 1974.

MATERIALS AND METHODS

1. *Experimental animals*

Male and female Donryu strain rats of 88 to 96 days old, weighing 200 to 300 g, were kept at room temperature and fed on standard commercial diet with or without 0.06% (W/W) 3′-Me-DAB and water *ad libitum*. The rats consumed an average of 15±5 g of food per day. Another group of male Donryu strain rats of 88 to 96 days old, were fed on a standard commercial diet, and injected intraperitoneally with 3′-Me-DAB (30 mg/kg body weight) once daily for 5 days.

2. *Subfractions of rat liver microsomes*

Fractions of plasma membranes, Golgi apparatus, and smooth and rough microsomes of the livers were prepared by the methods of Fleischer et al. (1969) and Peters (1962), after removing free polyribosomes by the method of Bloemendal et al. (1967). All procedures were carried out at 0 to 4 C.

3. *Enzyme assays*

Reported procedures were used to determine the contents of cytochrome P-450 (Ichikawa and Mason, 1972) and cytochrome b_5 (Garfinkel, 1958), the activities of succinate-cytochrome c reductase (Tisdale, 1967), glucose 6-phosphatase (Swanson, 1955), galactosyltransferase (Babad and Hassid, 1956), and 5′-nucleotidase (Michell and Hawthorne, 1965) and the *p*-hydroxylation of anilines (Brodie and Axelrod, 1948) and demethylation of *p*-nitroanisole (Netter and Seidel, 1964). NADPH- and NADH-ferricyanide reductase activities were determined using 0.6 mM ferricyanide as an electron acceptor. Enzyme activities were determined at 25 C.

4. *Instruments*

Light absorption spectra were measured with a Cary, Model 14 recording spectrophotometer. Electron paramagnetic resonance (EPR) absorption spectra were measured with a Varian spectrometer, Model V-4500-10A, equipped with 100 kc/sec field modulation and control unit, Model V-4560. Quartz sample tubes, with an inner diameter of 3.5 mm, were used in a liquid hydrogen dewar.

5. *Chemical analysis*

To avoid interference from nucleic acid, protein content was determined by the biuret reaction (Gornall et al., 1949), after addition of 1% sodium cholate to the sample to decrease turbidity. Crystalline bovine serum albumin was used as a standard. The complication of increase in the absorption at 540 nm in the biuret reaction due to heme in the test samples was avoided by using a reference containing pigment in 4% sodium hydroxide. The RNA content was determined by extraction with 5% trichloroacetic acid and analysis with orcinol (Schneider, 1957).

Standard diet with or without 0.06% 3′-Me-DAB was obtained from Oriental Yeast Co. All other reagents were of the highest purity available commercially.

RESULTS

1. *Wet weight of liver*

Administration of diet containing 3′-Me-DAB to Donryu strain rats resulted in almost 100% incidence of hepatomas in 6 months in male rats and in 9 months in female rats. The occurrence of hepatomas was shown histologically. After these periods, the wet weight of the liver was 3 to 5 times more than that of normal rats, as shown in Table 1.

TABLE 1. *Wet weight of livers of normal male rats and those treated with 3′-Me-DAB for 6 months*[a]

Liver	Wet weight (g)
Normal	12.6± 0.2
3′-Me-DAB-treated	41.3±10.0

[a] Values are means and standard deviations of values in 9 to 19 animals.

2. *Subfractions of liver microsomes*

Table 2 shows that the fractions of plasma membranes, Golgi apparatus and smooth and rough microsomes from normal rats and those treated with 3′-Me-DAB for 6 months were separated relatively sharply, judging from the activities of the marker enzymes (5′-nucleotidase for plasma membranes, galactosyltransferase for the Golgi apparatus and glucose 6-phosphatase activity and RNA content for smooth and rough microsomes). Data on the relative specific activities of the marker enzyme

TABLE 2. *Distributions of marker enzyme activities in submicrosomal fractions of livers of normal male rats and those treated with 3′-Me-DAB for 6 months*[a]

Fraction	5′-Nucletidase[b]	Galactosyltransferase[b]	Glucose 6-phosphatase[b]	RNA[c]
A) Normal				
Plasma membranes	134.5±15.4 (100.0)	0.04±0.01 (1.6)	47.0±12.0 (14.7)	<0.01 (<10.0)
Golgi apparatus	40.2±7.5 (29.9)	2.45±0.50 (100.0)	95.5±15.4 (29.8)	0.02 (20.0)
Smooth microsomes	20.5±9.0 (15.2)	0.25±0.05 (10.2)	170.0±25.4 (53.0)	0.02±0.01 (20.0)
Rough microsomes	5.4±2.0 (4.0)	0.15±0.05 (6.1)	320.5±27.5 (100.0)	0.10±0.01 (100.0)
Whole microsomes	14.5±5.6 (10.8)	0.25±0.05 (10.2)	250.0±30.5 (78.0)	0.05±0.01 (50.0)
B) 3′-Me-DAB-treated				
Plasma membranes	105.5±10.4 (100.0)	<0.06 (<2.0)	10.3±5.0 (5.1)	<0.01 (<6.7)
Golgi apparatus	35.5±10.0 (33.7)	3.00±0.50 (100.0)	30.5±10.4 (15.2)	0.03±0.01 (20.0)
Smooth microsomes	15.4±5.5 (14.6)	0.25±0.05 (8.3)	90.4±25.0 (45.1)	0.05±0.02 (33.3)
Rough microsomes	2.5±1.0 (2.4)	0.20±0.05 (6.7)	200.5±30.4 (100.0)	0.15±0.02 (100.0)
Whole microsomes	9.5±0.5 (9.0)	0.28±0.05 (9.3)	154.0±25.5 (76.8)	0.07±0.02 (46.7)

[a] Values are means and standard deviations of values in 5 to 10 rats. Values in parentheses are percentages of the maximal specific activities of the enzymes or maximal amount of RNA.
[b] *n* moles/min/mg protein.
[c] mg/mg protein.

in each fraction are shown in Table 2 as percentages of the maximal specific activities of these enzymes.

3. *Cytochromes and related enzymes in liver microsomes*

Table 3 shows the contents of cytochrome P-450 and cytochrome b_5 and related enzyme activities in submicrosomal fractions of the livers of normal rats and those treated with 3′-Me-DAB for 6 months. The cytochrome P-450 and b_5 contents of the smooth microsomes and Golgi apparatus decreased most on 3′-Me-DAB administration, followed by the cytochrome content of the rough microsomes. This treatment did not affect the NADPH- and NADH-ferricyanide reductase activities of the submicrosomal fractions, but reduced the activities for hydroxylation and demethylation of aniline, *o*-chloroaniline and *p*-nitroanisole of the submicrosomal fractions to about 15% of the normal levels.

Table 3 shows the relative specific activities of the submicrosomal fractions of 3′-Me-DAB-treated liver as percentages of those of the submicrosomal fractions of normal liver. These results show that the contents and activities of these enzymes, cooperatively functioning as a mixed function oxidase system, diminished or disappeared during liver carcinogenesis induced by 3′-Me-DAB.

4. *EPR spectra of liver microsomes*

Figure 1 shows that almost all the ferri-cytochrome P-450 and ferri-cytochrome b_5 of liver microsomes of normal and 3′-Me-DAB-treated

TABLE 3. *Components and activities of mixed function oxidase systems of submicrosomal fractions of*

Fraction	Cyt. P-450[b]	Cyt. b_5[b]	NADPH-ferricyanide reductase[c]
A) Normal			
Plasma membranes	0.00	0.00	7.1±2.5
Golgi apparatus	<0.04	0.45±0.10	25.4±2.4
Smooth microsomes	0.65±0.15	0.59±0.10	77.5±2.0
Rough microsomes	0.60±0.15	0.57±0.10	70.0±2.0
Whole microsomes	0.52±0.14	0.44±0.10	65.5±2.5
B) 3′-Me-DAB-treated			
Plasma membranes	0.00 (—)	0.00 (—)	5.5±1.4 (77.5)
Golgi apparatus	<0.01 (25.0)	0.10±0.10 (22.2)	20.5±2.7 (80.7)
Smooth microsomes	0.20±0.05 (30.8)	0.14±0.10 (23.7)	55.2±2.5 (71.2)
Rough microsomes	0.32±0.05 (53.3)	0.28±0.07 (49.1)	67.4±2.5 (96.3)
Whole microsomes	0.24±0.05 (64.2)	0.20±0.05 (45.5)	57.0±2.9 (87.0)

[a] Values are means and standard deviations of values in 5 to 10 rats. Values in parentheses are percentages of the specific activities of enzymes of normal submicrosomal fractions.

rats were in low spin forms at 20 K. Less than 5% of the ferri-cytochrome P-450 was in a high spin form. Similar results were obtained with liver microsomes from female rats fed on diets with and without 3′-Me-DAB for 9 months. The g values of ferri-cytochromes P-450 and b_5 of liver microsomes were the same in normal and 3′-Me-DAB-treated rats, although the contents of cytochromes P-450 and b_5, as shown in the case of cytochrome P-450 (so-called microsomal Fe_x) of other hepatomas (Nebert and Mason, 1963), decreased during carcinogenesis. The g values and orbital energy differences of d_z of heme-irons of ferri-

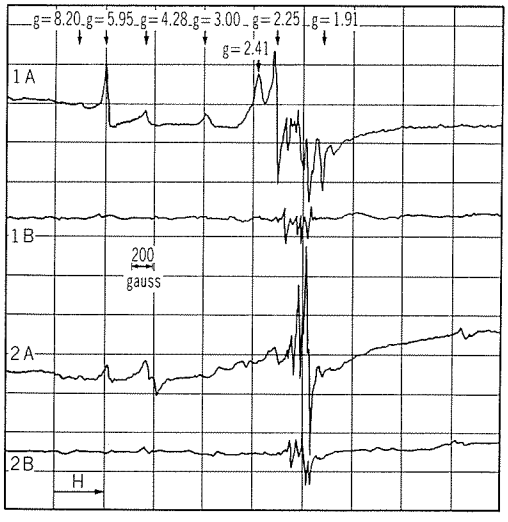

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FIGURE 1. EPR spectra of microsomes of livers from normal male rats and those treated with 3′-Me-DAB for 6 months. The EPR absorption spectra are shown in the region between g=1.0 and g=9.0. The microsomes were suspended in 0.1 M K-phosphate buffer, pH 7.5 at 25 C and frozen at 77 K. Spectra were measured at 20 K with an EPR spectrometer. Modulation amplitude, 12.5 gauss; microwave power, 3 mw; microwave frequency, 9.15 GHz; scanning rate, 200 gauss per min; temperature, 20 K; increased from low to high magnetic field.

1, Normal microsomes; 2, 3′-Me-DAB-induced microsomes; (A), oxidized form; (B), dithionite-reduced form.

the livres of normal rats and those treated with 3'-Me-DAB for 6 months[a]

NADH-ferricyanide reductase[c]	Hydroxylation[c] of		Demethylation[c] of *p*-nitroanisole
	o-chloroaniline	aniline	
24.3±2.5	0.00	0.00	0.00
750.4±33.4	0.07±0.02	0.05±0.02	0.04±0.01
1846.0±45.5	1.52±0.15	1.00±0.15	0.73±0.02
1245.5±49.4	0.34±0.15	0.94±0.15	0.65±0.02
1325.0±45.0	1.44±0.15	0.97±0.14	0.68±0.04
20.2±2.7 (83.1)	0.00 (−)	0.00 (−)	0.00 (−)
595.2±40.7 (79.3)	<0.02 (<28.6)	<0.01 (<20.0)	<0.01 (<25.0)
1597.5±54.5 (86.5)	0.25±0.10 (16.5)	0.14±0.05 (14.0)	0.13±0.05 (17.8)
1207.3±50.4 (96.9)	0.27±0.15 (20.2)	0.15±0.05 (16.0)	0.10±0.05 (15.4)
1474.5±50.0 (111.3)	0.24±0.10 (16.7)	0.14±0.05 (14.4)	0.12±0.04 (17.7)

[b] *n* moles/mg protein (abbreviations: Cyt. P-450, cytochrome P-450; Cyt. b_5, cytochrome b_5).
[c] *n* moles/min/mg protein.

TABLE 4. *Observed and calculated g values and orbital energy difference of d_ξ of the heme-irons of ferri-cytochrome P-450 of liver microsomes from normal and 3'-Me-DAB-treated rats*[a]

Liver microsomes	Ferri-cytochrome P-450 in low spin form				
	g Values			Orbital energy differences of d_ξ of heme-irons	
	g_x	g_y	g_z	$E_\xi - E_\eta$	$E_\xi - E_\zeta$
Normal	1.91 (1.90)	2.25 (2.24)	2.41 (2.40)	1864 cm^{-1}	2740 cm^{-1}
3'-Me-DAB-treated	1.91 (1.90)	2.25 (2.24)	2.41 (2.40)	1864 cm^{-1}	2740 cm^{-1}

[a] The microsomes (20 mg protein/ml) were suspended in 0.1 M K-phosphate buffer at pH 7.5 and frozen at 20 K. The orbital energy difference of d_ξ orbitals and calculated g values were calculated from the observed g values by the method of Kotani (1961). The orbital coupling constant was assumed to be 400 cm^{-1} (Kotani, 1961). The values in parentheses are calculated from the observed g values.

cytochrome P-450 are summarized in Table 4. The spin forms of ferri-cytochrome P-450 of rat liver microsomes were not affected by addition of 3'-Me-DAB in vitro. Moreover, when 3'-Me-DAB (30 mg/kg body weight) was injected intraperitoneally into male rabbits or rats daily for 5 days, the contents of cytochromes P-450 and b_5 were not affected and the high spin form of ferri-cytochrome P-450 did not increase. The results are shown in figure 2. The difference spectra of the dithionite-reduced cytochrome P-450 complex with CO minus the dithionite-reduced cytochrome P-450 obtained from normal and 3'-Me-DAB-

treated rat livers had an absorption peak of the Soret band at 450 nm. In this, it differed from liver cytochrome P-448 of 3-methylcholanthrene-treated rats and rabbits (Peisach and Blumberg, 1970).

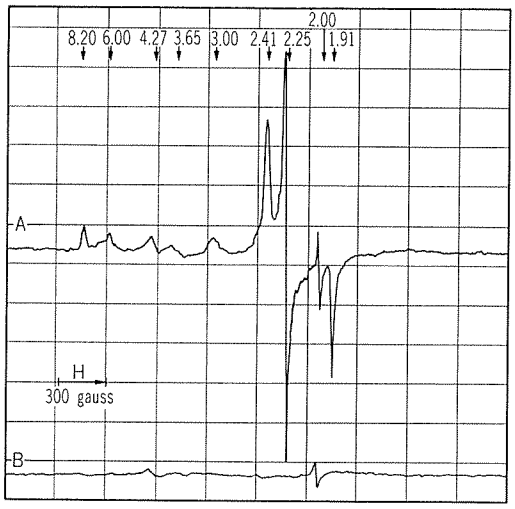

FIGURE 2. EPR spectra of liver microsomes of male rats injected with 3′-Me-DAB for 5 days.
Conditions for the EPR were the same as for Fig. 1. (A), Oxidized form; (B), Dithionite-reduced form.

DISCUSSION

Treatment of rats with 3′-Me-DAB resulted in greater reduction of the activities of hydroxylation and demethylation of the *p*-position of anilines and *p*-nitroanisole than in the contents of cytochromes P-450 and b_5 and of the NAD(P)H-ferricyanide reductase activity. These results suggest that the cooperative function of these components as a cytochrome P-450-linked mixed function oxidase system of liver microsomes may be disturbed during liver carcinogenesis. The mixed function oxidase systems of the smooth endoplasmic reticulum and Golgi apparatus also decreased considerably, more than that of the rough endoplasmic reticulum, on administration of 3′-Me-DAB. This decrease in rats is possibly influenced by the turnover of components of microsomal mixed function oxidase systems during liver carcinogenesis induced by 3′-Me-DAB, in addition to the effect of carcinogenesis on the binding of polyribosomes to the rough endoplasmic reticulum (Uenoyama and Ono, 1972).

The rate of incidence of hepatomas in rats induced by 3′-Me-DAB is considerably affected by differences in sex (Bürki et al., 1973), age, species (Peisach et al., 1972), strain and type of tissue. The reason for the sex difference is still unknown. However, judging from the activity of polycyclic hydrocarbon hydroxylase in the liver of male and female mice (Bürki et al., 1973), the enzyme systems in the liver of female rats may be able to metabolize 3′-Me-DAB better than those of male rats (Ichikawa, unpublished data). Oxidized and high spin forms of cytochrome P-450 (or cytochrome P-448) are affected by carcinogens such as 3-methylcholanthrene and 3, 4-benzpyrene (Peisach et al., 1972), but not by 3′-Me-DAB.

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