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Spectrophotometric Analysis of Cytochromes in Rat Liver during Carcinogenesis

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SUMMARY

The cytochrome content of liver mitochondria and microsomes during the carcinogenesis of rat liver by both 3'-methyl-4-dimethylaminoazobenzene (3'-DAB) and 4'-methyl-4-dimethylaminoazobenzene (4'-DAB), and of 3'-DAB-induced hepatomas, was determined spectrophotometrically at room and liquid nitrogen temperatures and was compared with that of normal liver. The cytochrome $a + a_3$ content in the livers of rats fed with the standard amounts of 3'-DAB or 4'-DAB began to decrease from the 16th week. At the 27th week the cytochrome content approached the level of the 3'-DAB-induced hepatoma, which is nearly one-half that in normal liver. Conversely, the content of cytochromes b, c_1, and c did not change significantly during liver carcinogenesis by 3'-DAB and 4'-DAB, or in the 3'-DAB-induced hepatoma. The ratio of cytochrome $a + a_3$ to cytochrome c of mitochondria in liver decreased from 1.1 to 0.6 in the precancerous state and finally reached 0.5 in the hepatoma. The microsomal cytochrome b_5 and P-450 content did not alter gradually during carcinogenesis but decreased abruptly in the hepatoma.

INTRODUCTION

In the 1st report of this series (10), we showed by spectrophotometric analysis that the cytochrome $a + a_3$, b, and c_1 content in various ascites hepatomas was much lower than that in normal liver, while the cytochrome c content was nearly the same. As a result, the ratio of cytochromes $a + a_3$, b, and c_1 to cytochrome c in the tumors was remarkably lower than that in normal liver. This characteristic of cytochrome composition in tumor cells was also demonstrated to be true in the minimum deviation hepatoma, as reported previously (5). Hiraga and Adachi (6) reported a low cytochrome $a + a_3$, b, c_1, and c content in melanoma, compared with that normal rat heart, rat liver, and Baker's yeast.

Moreover, we found that the cytochrome composition of tumor-bearing rat livers resembled, although not remarkably, that of tumors themselves (5). In the study described in this paper, we determined the cytochrome content in livers of rats fed 3'-DAB,[2] a potent carcinogen, and 4'-DAB, a mild carcinogen, as well as that in resulting hepatomas.

MATERIALS AND METHODS

Animals and Diets. Sprague-Dawley male rats weighing 180 ± 15 g were divided into 3 groups of 20 rats each. The 1st group was used as a control and was fed the standard M-diet (protein content, 24.2%) from the Oriental Yeast Co., Osaka, Japan. The 2nd and 3rd groups were fed the carcinogenic M-diet (Oriental yeast), which contains 0.06% 3'-DAB and 4'-DAB, respectively. Both the carcinogen-containing diet and the M-diet containing no carcinogen were given until the 16th week; then all 3 groups were changed to the CM-diet (protein content, 29.8%). The daily carcinogen uptake during the treatment period was about 12 mg/rat.

Preparation of Mitochondria and Microsomes. The animals were killed by decapitation and the livers were perfused with cold 0.9% NaCl solution via the portal vein. Five g of perfused liver tissue were minced by scissors in a 45-ml solution containing 0.25 M sucrose, 0.1 mM EDTA, and 10 mM potassium phosphate buffer, pH 7.4. The resulting suspension was homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle at 0-4°.

The tissue debris and nuclei were removed from the homogenate (50 ml) by centrifugation at 600 × g for 5 min, and the mitochondrial fraction was sedimented at 8,000 × g for 8 min. The mitochondrial pellets were washed twice and suspended in the same medium to the final volume of 10 ml (0.5 g tissue equivalent per ml). All these centrifugations were performed with the fixed-angle rotor (R_{max} = 11.0 cm) of a Tominaga 5-62 type centrifuge. Sixteen ml (corresponding to 1.6 g tissue equivalent, since 16 ml were used of 50 ml of 5 g tissue homogenate) of the resulting supernatant fluid were further centrifuged at 105,000 × g for 60 min with the fixed-angle rotor (R_{max} = 7.05 cm) of a Beckmann ultracentrifuge. The pellet was suspended in the same medium to 10 ml (0.16 g tissue equivalent per ml) as the microsomal sample. All procedures were conducted at 0 to 4°.

When a hepatoma was found in the liver, it was removed, together with the surrounding part of the tissue. The remaining part of the liver tissue was used in the same way as were the carcinogen-treated livers without hepa-

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[2] The abbreviations used are: 3'-DAB, 3'-methyl-4-dimethylaminoazobenzene; 4'-DAB, 4'-methyl-4-dimethylaminoazobenzene.

toma. The cancerous, noncancerous, and necrotic parts were carefully separated, and the resulting part was used for the assay of cytochrome content of the hepatoma, after it had been treated as described above.

Determination of Cytochrome Content. For determination of cytochrome $a + a_3$, c, and c_1 content in the mitochondrial suspension, the difference spectra between the anaerobic and aerobic states were taken at room and liquid nitrogen temperatures (77°K), as described previously (5, 10). However, anaerobiosis was achieved by the respiration with the mixture of succinate and glutamate (5 mM) as substrate. For determination of cytochrome b, the measure sample was treated with antimycin A (0.5 nmole/mg mitochondrial protein) in the presence of succinate (5 mM) and glutamate (5 mM) and then aerated for 30 sec. The reference sample was treated with rotenone (5 μM) in the aerobic state, and the difference spectra were measured at room temperature only.

For determination of cytochromes b_5 and P-450 in the microsomal suspension, the difference spectra were obtained between the presence and absence of NADH (0.5 mM) in the aerobic condition, and between the reduced (with sodium dithionite) and oxidized (aerobiosis) states in the presence of CO, as described in the preceding paper (5). Each calculation of cytochrome content was carried out by the method described previously (5, 10).

In order to determine the protein content, the biuret method of Garnall *et al.* (4) was used with bovine serum albumin as standard. However, a 4-fold concentration of copper sulfate and Rochelle salt was used to obtain better linearity.

RESULTS

Change in Concentration of Respiratory Cytochromes during Carcinogenesis. Chart 1 shows representative difference spectra of liver mitochondria of rats fed 3'-DAB. Considerable differences exist between these curves. These differences are remarkable especially around γ bands between the normal and treated rats. Similar changes were observed in the case of 4'-DAB treatment (Curve 4), although no hepatoma was induced.

The difference spectra shown on Chart 1 were obtained on mitochondria from each batch of 2 to 4 rats at 4, 10, 16, 20, 22, and 27 weeks with 3'-DAB treatment and at 4, 10, 16, 22, and 27 weeks with 4'-DAB treatment; they were also obtained on 3'-DAB-induced hepatomas. The changes in cytochrome concentrations during carcinogensis are illustrated in Chart 4 and Table 1.

As shown in Chart 4*A*, the concentrations of cytochrome $a + a_3$ began to decrease at the 16th week and became about one-half the normal value after the 22nd week, in the case of both 3'- and 4'-DAB. The change in cytochrome b content was not remarkable until the 27th week. The concentrations of cytochrome $c + c_1$ increased slightly at the beginning of 4'-DAB feeding and then gradually decreased from the 16th week. At the 16th week, when the concentration of cytochromes $a + a_3$ began to decrease, the livers of rats fed 3'-DAB were characterized morphologically as "fatty." The

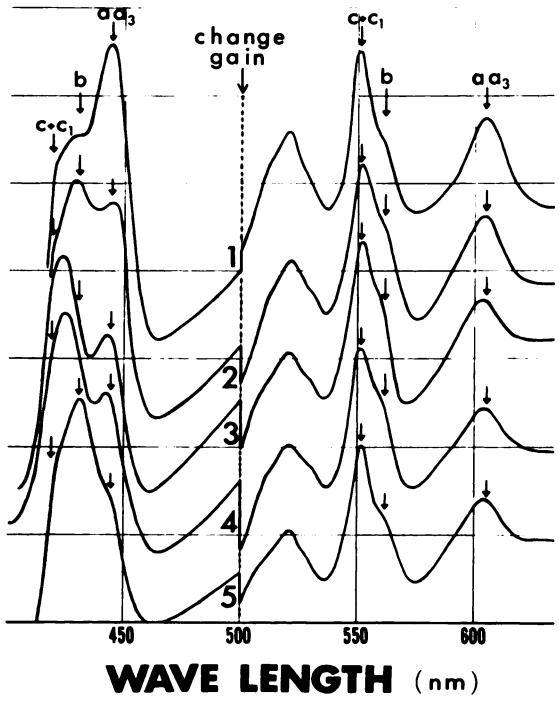

Chart 1. Typical examples of the room-temperature difference spectra between anaerobic and aerobic states of liver mitochondria from rats during treatment with 3'- and 4'-DAB. Conditions are given in "Materials and Methods." One section of the figure corresponds to 0.125 A in the range of wavelength shorter than 500 nm, and 0.05 A in the longer range. *Curve 1*, liver mitochondria from normal rat at zero week (7.0 mg protein per ml); *Curve 2*, those from rat treated with 3'-DAB for 16 weeks (7.8 mg protein per ml); *Curve 3*, those from rat treated with 3'-DAB for 16 weeks and kept with CM-diet for 6 weeks (8.1 mg protein per ml); *Curve 4*, those from rat treated with 4'-DAB for 16 weeks and kept with CM-diet for 6 weeks (8.0 mg protein per ml); *Curve 5*, mitochondria from 3'-DAB-induced hepatoma (7.5 mg protein per ml). *Arrows* (from *right* to *left*), positions of α-bands of cytochromes aa_3, b, and $c + c_1$ and those of γ-bands of cytochromes aa_3, b, and $c + c_1$.

hepatoma usually began to appear after the 22nd week, but in 1 case it appeared at the 14th week of 3'-DAB treatment. In the case of 4'-DAB treatment, no morphological changes were observed by light-microscopic observation until the 27th week, yet the cytochrome $a + a_3$ content decreased.

In order to determine the change of cytochromes more precisely, the difference spectra at liquid nitrogen temperature were taken on a few mitochondrial preparations at each stage of carcinogenesis. Representative spectra are shown in Chart 2. Table 1 shows the average value of cytochrome c concentrations calculated from both room and low-temperature spectra (as in Charts 1 and 2), as well as the ratio of each respiratory cytochrome.

Microsomal Cytochrome Content during Carcinogenesis. Chart 3 shows the spectra of cytochromes b_5 and P-450. These spectra were used for calculation of the concentrations summarized in Table 2. After the 22nd week, the cytochrome b_5 content did not change significantly in the precancerous livers of 3'- and 4'-DAB-treated rats. However, the cytochrome b_5 content in the hepatoma was only about 35% of that in the normal liver. The concentration of cytochrome P-450 decreased to a certain degree in the

Table 1

Concentrations of cytochrome c and relative concentration of respiratory cytochromes in the livers of normal and carcinogen-fed rats

Material	Mean cytochrome *c* concentration[a] (nmoles/mg protein)	Ratio[b]			
		c	$a + a_3$	*b*	c_1
Normal liver					
Wk 0[c] (5)[d]	0.163 (0.144–0.193)[e]	1	1.1	0.5	0.6
Wk 10 (2)	0.165 (0.150–0.180)	1	1.1	0.5	0.6
Wk 16 (2)	0.170 (0.163–0.177)	1	1.1	0.5	0.7
Wk 22 (4)	0.168 (0.154–0.181)	1	1.1	0.5	0.7
Wk 27 (2)	0.173 (0.160–0.186)	1	1.1	0.5	0.7
3′-DAB-treated liver					
Wk 4 (3)	0.163 (0.157–0.171)	1	1.3	0.5	0.7
Wk 10 (2)	0.171 (0.148–0.187)	1	1.1	0.5	0.7
Wk 16 (2)	0.161 (0.155–0.165)	1	0.8	0.6	0.7
Wk 20 (2)	0.132 (0.117–0.155)	1	0.9	0.7	0.7
Wk 22 (3)	0.144 (0.126–0.177)	1	0.8	0.7	0.7
Wk 27 (3)	0.150 (0.115–0.173)	1	0.6	0.7	0.7
3′-DAB hepatoma					
(5)	0.171 (0.154–0.191)	1	0.5	0.7	0.6
4′-DAB					
Wk 4 (3)	0.207 (0.184–0.223)	1	1.0	0.5	0.6
Wk 10 (2)	0.193 (0.186–0.200)	1	0.9	0.4	0.6
Wk 16 (2)	0.146 (0.128–0.163)	1	0.9	0.5	0.8
Wk 22 (3)	0.122 (0.097–0.164)	1	0.8	0.7	0.7
Wk 27 (4)	0.154 (0.128–0.173)	1	0.7	0.5	0.6

[a] Calculated from the room and low temperature spectra.

[b] Calculated with the use of both room and low temperature spectra (see Ref. 5). Cytochrome *c* concentration is taken as 1.

[c] Rats at the starting point (see "Materials and Methods").

[d] Column 1: numbers in parentheses, number of animals used.

[e] Column 2: numbers in parentheses, range (minimum to maximum value).

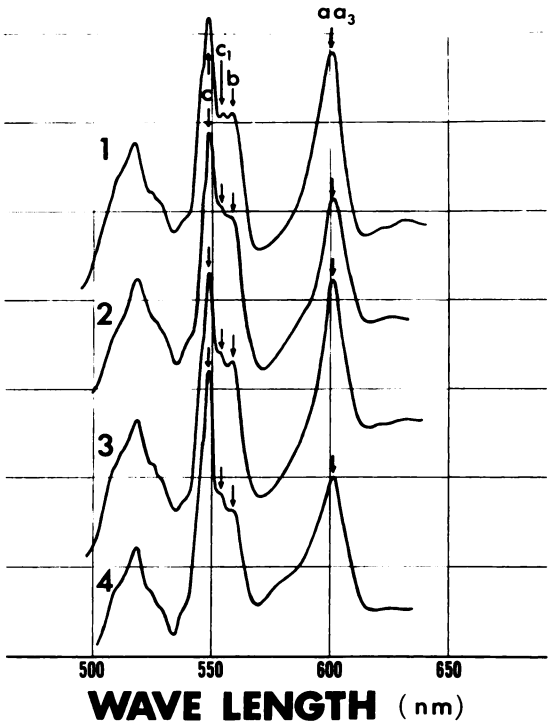

Chart 2. Low-temperature "anaerobic minus aerobic" difference spectra of mitochondria from livers of normal, 3′-DAB-treated, and 4′-DAB-treated rats, and from 3′-DAB-induced hepatoma. *Curve 1*, normal rat liver mitochondria at zero week (7.0 mg protein per ml); *Curve 2*, mitochondria at the 27th week of 3′-DAB treatment (7.5 mg protein per ml); *Curve 3*, mitochondria of a rat at the 26th week of 4′-DAB treatment (7.7 mg protein per ml); *Curve 4*, mitochondria from the 3′-DAB-induced hepatoma (7.5 mg protein per ml). *Arrows* (from *right* to *left*), position of α (or α_1) peaks of cytochromes $a + a_3$, *b*, c_1, and *c*.

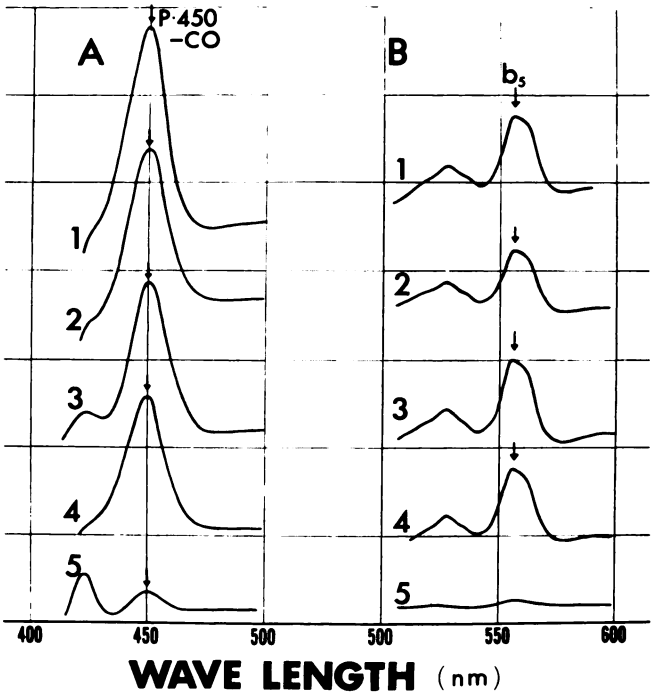

Chart 3. Representative difference spectra of microsomes from normal and precancerous livers and hepatoma showing the amounts of cytochrome P-450 and b_5. *A*, Difference spectra between the presence and absence of dithionite in the aerobic suspension of microsomes containing 0.1 mM NADH and treated with CO for 60 sec. One section of the figure corresponds to 0.06 A. *B*, Difference spectra between the presence and absence of NADH (0.2 mM) in the aerobic suspension of microsomes. One section of figure corresponds to 0.02 A. *Curve 1*, normal rat liver microsomes of zero week (1.7 mg protein per ml); *Curve 2*, microsomes (1.5 mg protein per ml) at the 22nd week of 3'-DAB treatment; *Curve 3*, microsomes (1.3 mg protein per ml) at the 27th week of 3'-DAB treatment; *Curve 4*, microsomes (1.0 mg protein per ml) at the 26th week of 4'-DAB treatment; *Curve 5*, microsomes (1.1 mg protein per ml) of the 3'-DAB-induced hepatoma.

precancerous liver and became about 15% of that in the normal liver.

DISCUSSION

As shown in Charts 1 and 4 and in Table 1, the concentration of cytochrome $a + a_3$ in mitochondria decreased remarkably, compared with that of cytochromes b, c_1, and c, during carcinogenesis with 3'- and 4'-DAB. In the last stage of carcinogenesis (after the 20th week), the cytochrome composition of the noncancerous part of livers became similar to that of hepatoma itself in about 80% of rats treated with 3'-DAB after the 22nd week. We found large morphological disturbances of these livers even if there was no hepatoma, a finding that has been reported by many investigators. Although the hepatoma was not induced at all in the case of 4'-DAB treatment, the cytochrome composition of liver was very similar to that with 3'-DAB treatment. It is supposed that administration of 4'-DAB under these conditions (rats fed 4'-DAB for 16 weeks, 12 mg/rat/day, and then maintained for about 11 weeks without the carcinogen) was enough to manifest a cytochrome composition similar to that of the 3'-DAB-induced hepatoma. According to Matsumoto and Terayama (7), the relative carcinogenic potency of 3'-DAB to 4'-DAB is about 10 to 1. The feeding of rats with 3'-DAB for 16 weeks was sufficient to permit detection of decreased cytochrome $a + a_3$ concentration in precancerous liver. It was found that, when rats were fed 3'-DAB for only 2 weeks (instead of 16 weeks, as in this study) and were kept for 25 weeks without carcinogen, the regimen was sufficient to change the cytochrome composition to a state similar to that of the hepatoma (Y. Ōyanagui and B. Hagihara, unpublished results). This treatment did not induce hepatoma at all until the 27th week.

In the case of various ascites tumors (10) and Morris hepatomas (5), there was a decrease, not only in cytochrome $a + a_3$ but also in cytochromes b and c_1, compared with that of normal liver. A similar although much less signifi-

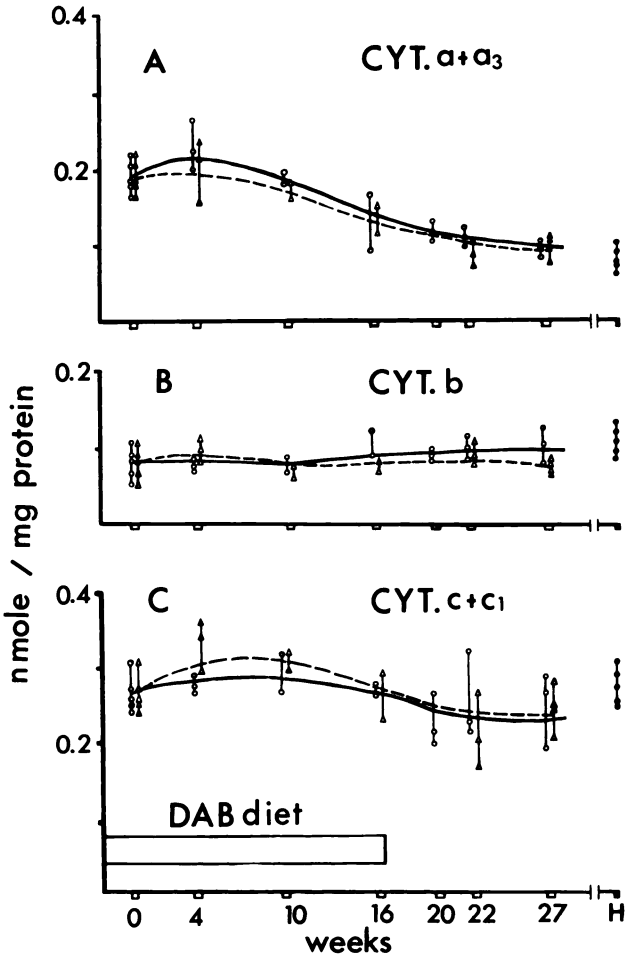

Chart 4. Change in respiratory cytochrome (*CYT.*) concentrations in mitochondria of rats during 3'- and 4'-DAB carcinogenesis. Conditions are described in "Materials and Methods." *A*, *B*, and *C*, gradual change of cytochromes $a + a_3$, b, and $c + c_1$, respectively. *Circles* and *solid line*, values for the 3'-DAB treatment; *triangles* and *dotted line*, values for the 4'-DAB treatment.

Table 2
Contents of microsomal cytochromes of precancerous livers and hepatoma

Material	Concentration (nmoles/mg protein)	
	b_5	P-450
Normal liver (5)[a]	0.34 ± 0.03[b]	0.85 ± 0.08
Precancerous liver 3'-DAB (22nd week) (3)	0.35 ± 0.03	0.68 ± 0.04
4'-DAB (22nd week) (3)	0.38 ± 0.04	0.52 ± 0.06
3'-DAB hepatoma (5)	0.12 ± 0.04	0.10 ± 0.05

[a] Numbers in parentheses, number of experiments.
[b] Mean ± S.E.

cant tendency was also observed in the livers of tumor-bearing rats (5). In the 3'-DAB hepatoma as well as in the liver during carcinogensis, however, the cytochrome b and c_1 contents were similar to that of normal liver. These differences may be due to the fact that this hepatoma is an originally induced cancer and not a continuously transplanted one, as in the case of other tumors. As the number of transplants from generation to generation increased, the change in the composition of cytochromes in Morris hepatomas also increased (N. Sato and B. Hagihara, unpublished results).

Decreased cytochrome $a + a_3$ content in precancerous livers and hepatomas may be related to the low copper content in the hepatomas (14, 16) and in 3'-DAB precancerous livers (1). It is well known that copper is an essential component of cytochrome $a + a_3$ (cytochrome oxidase), and there are findings about the decreased cytochrome oxidase content caused by copper-deficient nutrition in animals (3, 15) and yeast (15).

As shown in Table 2, the decrease in concentration of microsomal cytochrome b_5 and P-450 was much greater in the hepatoma, compared with the reduction of respiratory cytochrome $a + a_3$ content in tumors. The concentrations of these cytochromes in the 3'-DAB-induced hepatoma, were lower than those in the slowly growing Morris hepatoma (5) but were higher than those in rapidly growing ascites hepatomas which usually contain little or none of these cytochromes (9-11). The decrease of microsomal enzyme activities (2, 8, 13), as well as protein content and glycogen content (9), may be related to the decrease of microsomal cytochromes. Despite the remarkably decreased concentrations of cytochromes b_5 and P-450 in the hepatoma, the liver during carcinogenesis still contains only slightly less than normal levels of these cytochromes. This differs also from the mode of change in mitochondrial respiratory cytochrome content.

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