

Effect of Phenobarbital and 3-Methylcholanthrene on Decrease in pH in Liver induced by 3'-Methyl-4-dimethylaminoazobenzene, of Partial Hepatectomy and Hepatotoxic Agents¹⁾

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(Received May 2, 1978)

We investigated whether 3-methylcholanthrene (3MC) or phenobarbital (PB) which inhibit hepatocarcinogenesis of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) block the decrease in intra- and extracellular pH (pHi and pHe) in the livers of rats on a diet containing 0.06% 3'-Me-DAB. In rats treated with 3MC or PB alone, pHe and pHi levels remained within normal limits. In rats on the 3'-Me-DAB diet, pretreatment with 3MC or PB prevented a pH decrease in the 1st and 5th weeks after inception of the 3'-Me-DAB diet. In partially hepatectomized rats on a basal diet, no pHe or pHi decrease in the regenerating liver was noted at 24 and 48 hr after hepatectomy. However, in partially hepatectomized rats on the 3'-Me-DAB diet, a significant pHi decrease in the regenerating liver was observed. In rats administered with 50 ppm diethylnitrosamine in place of drinking water, liver nodules developed. However, pHe and pHi dropped only slightly. The administration of CCl₄ or bromobenzene effected no significant decrease in pHe or pHi, however, a dose-response correlation was found to exist for CCl₄.

Keywords—3'-Me-DAB; intracellular pH; liver damage; 3-methylcholanthrene; phenobarbital; diethylnitrosamine; regenerating liver; CCl₄

To study the effect of foreign compounds on the physiological condition of organs *in vivo*, we previously examined the change in liver pH of rats treated with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), a hepatotoxic agent.³⁾ We found that the intracellular pH (pHi) in the liver of rats fed a diet containing 3'-Me-DAB decreased with prolongation of the feeding period, while the pHi in the liver of rats fed a diet containing 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), which is an analog of 3'-Me-DAB and not hepatocarcinogenic under normal conditions, did not decrease. It has been reported that the administration of polycyclic hydrocarbons or barbiturates inhibits 3'-Me-DAB-induced hepatocarcinogenesis⁴⁾ and that phenobarbital (PB) or 3-methylcholanthrene (3MC) inhibits azo dye carcinogenesis only when these drugs are administered prior to and simultaneous with azo dyes. Therefore, we studied the effect of PB or 3MC pretreatment on the decrease of pHi in rats on a 3'-Me-DAB diet. Since 3'-Me-DAB is known to stimulate cell proliferation in the liver during hepatocarcinogenesis,⁵⁾ we used partially hepatectomized rats to determine whether cell proliferation effects a decrease in pHi and to examine the degree of pHi changes. Furthermore, we used diethylnitrosamine (DENA), CCl₄, and bromobenzene to determine whether the pHi changes in rats treated with these foreign compounds is similar to the 3'-Me-DAB induced

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changes. Metabolized 3'-Me-DAB is conjugated with glucuronic and sulphuric acids, and subsequently becomes covalently bound to cellular protein.⁶⁾ DENA is hydroxylated on an α C-atom at first, dealkylated, and decomposes to the respective ethyl cation, an ultimate alkylating carcinogen.⁷⁾ The hepatotoxicity of CCl_4 has been attributed to the reaction of the free radical metabolite with lipid and protein constituents of the endoplasmic reticulum,⁸⁾ while the active intermediate of bromobenzene is an epoxide.⁹⁾ Since these four compounds effect liver damage in different ways, we investigated whether these hepatotoxins actions result in different physiological liver conditions.

Materials and Methods

Eleven week-old and 7 week-old male Donryu rats weighing about 200 g and 140 g, respectively, at the beginning of the experiments, were used. The cube diet (Oriental MF) was used as the basal diet, and the 3'-Me-DAB diet contained 0.06% of 3'-Me-DAB in this basal diet. Food and water were provided *ad libitum*. pHe was measured at 37° with a Blood Micro System, type BMS, in conjunction with Acid-Base Analyzer, type PHM 72 (Radiometer, Copenhagen) and pHi was determined by the DMO method.³⁾

Treatment with PB and 3MC—Sixty rats were injected intraperitoneally for 1 or 3 days with a daily dose of 80 mg/kg of PB in saline solution and 28 with a single dose of 20 mg/kg of 3MC in olive oil solution. At 24 and 48 hr, after the final injection, 24 rats from either group were killed and pHe and pHi values measured. At various periods after the final injections, 48 rats were killed and the activity of azo reductase in the liver determined. In the remaining 16 rats, the basal diet was changed 24 hr after the last injection to the semi-synthesized 3'-Me-DAB diet. At different times after inception of the 3'-Me-DAB diet, rats were sacrificed and pHe and pHi determined.

Azo Reductase Assay—Azo reductase activity was determined by the slightly modified method of Ishidate *et al.*,⁴⁾ using 9000 g supernatant of rat liver. The PB or 3MC treated rats were decapitated, bled, the livers removed, homogenized in 4 volumes of 0.75% KCl solution and centrifuged for 10 min at 9000 g. Azo reductase assay was performed in an incubation medium containing in a total volume of 3.1 ml; 0.1 M potassium phosphate buffer (pH 7.4), 120 μmol of nicotinamide, 9 μmol of glucose 6-phosphate, 139 μmol of NAD, 131 μmol of NADP, 70 μmol of KCl, 10 μmol of MgCl_2 , 1 ml of freshly prepared 9000 g supernatant, and 6.3 μmol of 3'-Me-DAB in 95% ethanol as substrate. After incubation at 37° for 45 min, 3 ml of 20% trichloroacetic acid in acetone-ethanol (1:1) was added, the mixture centrifuged and the supernatants were spectrophotometrically measured at 525 nm. Azo reductase activity was expressed in terms of decrease of the substrate per mg of the 9000 g supernatant protein during 45 min. The amount of protein was measured according to Lowry *et al.*¹⁰⁾

Partial Hepatectomy—Partial hepatectomy was performed according to the method of Higgins and Anderson.¹¹⁾ For two-thirds hepatectomy, the two central and left lateral lobes were removed. Penicillin was used as antiseptic. In sham operations, the lobes were lifted from the peritoneal cavity and immediately replaced. In 10 rats, the regenerating liver was used to determine pHe and pHi at 24 and 48 hr after the operation. The remaining hepatectomized rats were fed the 3'-Me-DAB diet for another week after the operation, sacrificed and pH was measured.

Treatment with Other Hepatotoxic Compounds—DENA was dissolved in water at 50 ppm and the solution was supplied to 35, 8 week-old rats *ad libitum* in place of drinking water. pHe and pHi were measured at different time intervals. CCl_4 was administered orally to 40 rats at various doses in an olive oil solution, and pHe and pHi were determined after 24 and 48 hr. Bromobenzene was dissolved in olive oil and administered intraperitoneally to 13 rats.

Results

Effect of PB or 3MC Pretreatment on 3'-Me-DAB induced pHi Decrease

pHe and pHi in rats treated with PB or 3MC are shown in Fig. 1. In general, the pH values obtained for treated rats were lower than those in the controls, although there was no

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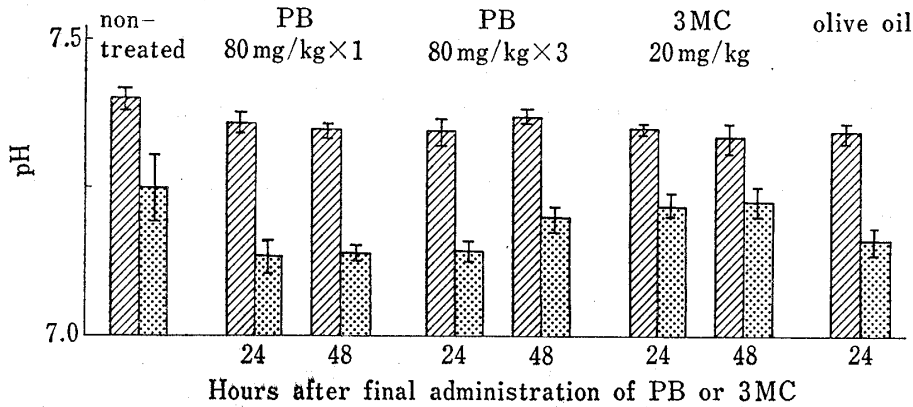


Fig. 1. Change in Intra- and Extracellular Liver pH in Rat treated with PB or 3MC

80 mg/kg phenobarbital was injected intraperitoneally daily for 1 (x1) or 3 (x3) days and 20 mg/kg 3-methylcholanthrene was injected once. Vertical bars indicate standard errors of the mean.

TABLE I. Comparison of Liver pH in Rat, Fed a 3'-Me-DAB Diet with and without PB or 3MC Pretreatment

Feeding period	Pretreatment	pHe	pHi
1 week	None	7.351 ± 0.015	6.999 ± 0.052
	PB	7.423 ± 0.006 ^{b)}	7.213 ± 0.021 ^{b)}
	3MC	7.348 ± 0.018	7.140 ± 0.027 ^{a)}
2 weeks	None	7.220 ± 0.039	6.936 ± 0.019
	PB	7.317 ± 0.019 ^{a)}	7.121 ± 0.015 ^{b)}
	3MC	7.286 ± 0.035	7.104 ± 0.029 ^{b)}
Normal		7.399 ± 0.010	7.174 ± 0.028

a) $p < 0.05$; b) $p < 0.01$; comparing non-pretreated with either PB or 3MC treated rats.

80 mg/kg PB was intraperitoneally injected daily for 3 days and 20 mg/kg 3MC was injected once. The feeding of the 3'-Me-DAB diet was initiated 24 hr after the final injection. The results represent the average ± S.E. for at least four animals.

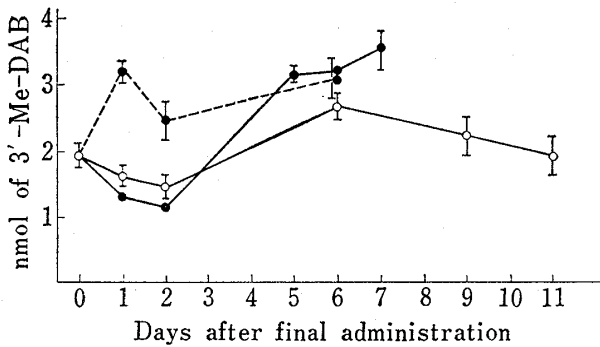


Fig. 2. Time Course of PB or 3MC-induced Azo Reductase Activity in Rat Livers

—●—, 3MC 20 mg/kg, *i.p.*, —●—, PB 80 mg/kg, *i.p.* for 3 days, —○—, PB 80 mg/kg, *i.p.* for 1 day. Activity is expressed in terms of decrease of 3'-Me-DAB per mg protein of the 9000 g supernatant per 45 min (nmol of 3'-Me-DAB). Each point represents mean ± S.E. for 3 or 4 rats.

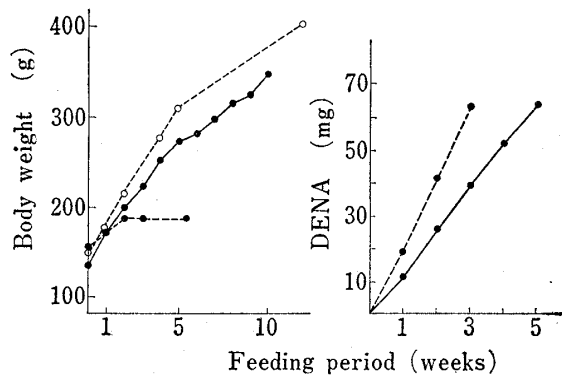


Fig. 3. Growth Curves of Rats Administered DENA and Integrated Intake of DENA

—○—, non-treated; —●—, DENA 50 ppm; —●—, DENA 100 ppm.

TABLE II. Change in Intra- and Extracellular Liver pH in partially Hepatectomized Rats

Time after Operation	No. of rats	pHe	pHi
24 hr	Sham	2	7.397
	True	5	7.379±0.019
48 hr	Sham	2	7.409
	True	5	7.403±0.013

The results represent the average±S.E.

significant difference between the two groups. On the other hand, pretreatment with PB or 3MC inhibited the 3'-Me-DAB induced decrease in the pHe and pHi for 1 and 5 weeks, and the inhibitory effect of PB on pHi decrease was greater than that of 3MC (Table I). PB or 3MC treatment effected an increase in azo reductase activity which lasted for more than 1 week (Fig. 2), indicating the duration of the action of the compounds. In the 5th week, pHi fell below values obtained in the 1st week, however, in comparison to pHi values in control livers, the values were markedly high.

pHi in partially Hepatectomized Rats and Effect of 3'-Me-DAB on Regenerating Liver pHi (Table II)

Twenty four hr after partial hepatectomy, pHe and pHi values decreased slightly but recovered to the normal range at 48 hr. In comparison with the pH values in sham-operated rats, no significant difference was detected. When rats were fed the 3'-Me-DAB diet for 5 days after partial hepatectomy, the values of pHe and pHi were 7.397 ± 0.020 and 6.955 ± 0.010 (mean±S.E., $n=3$), respectively.

Effect of DENA on Rat Liver

As shown in Fig. 3, at 50 ppm body weight increased smoothly, although the increase was slightly less than in controls. Since administration of 100 ppm inhibited growth completely after 2 weeks, a concentration of 50 ppm DENA was used in the drinking water. The change in pH during DENA administration illustrated Fig. 4. After 1 week pHe decreased, slightly recovered after 3 weeks, decreased again and remained below normal for the remainder of the experimental period. pHi decreased after 5 and 7 weeks and remained slightly below normal. A few small nodules were detected on the liver surface after 9 weeks, and they increased in number and size with prolongation of the feeding period. By week 19, the nodules had increased in size so that pH values could be determined at the center and at a periportal sites.

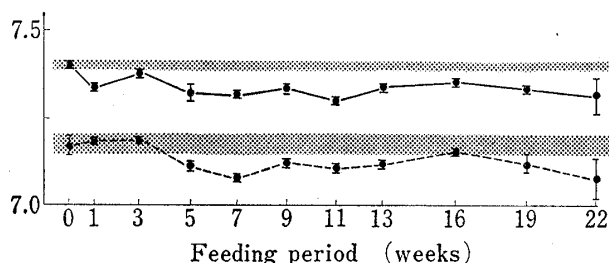


Fig. 4. Time Course of Intra- and Extracellular Liver pH in Rats Fed DENA Solution at 50 ppm

—, pHe; ---, pHi; [shaded] normal range.
Each point except the one at 0 week represents mean±S.E. for 3 or 4 rats.

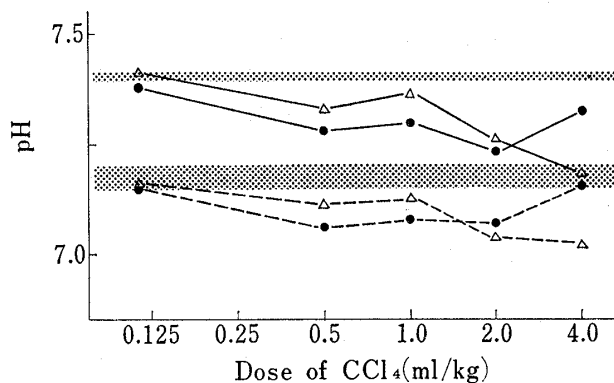


Fig. 5. Change in Intra- and Extracellular Liver pH in Rats orally Administered with CCl_4

—, pHe; ---, pHi; ● 24 hr and △ 48 hr after administration, [shaded] normal range.
Each point represents the mean for at least 3 rats.

pH values at those 2 sites were not identical, however, they were lower than pH values obtained in the rest of the liver.

Change in pH induced by the Administration of CCl_4 or Bromobenzene

pH was determined 24 and 48 hr after the administration of CCl_4 (Fig. 5). At dosages of less than 2.0 ml/kg, increasing the dose effected a decrease in pHe and pHi, and pHe and pHi showed lower values at 24 hr than at 48 hr. On the other hand, pHi in rats treated with 2.0 ml/kg or above was higher at 24 hr than 48 hr. More than half of the rats administered 4.0 ml/kg died and pHe and pHi values in the remaining rats were much higher at 24 hr than at 48 hr. In general, both pHe and pHi decreased with the dosage increase and the degree of decrease in pHe was larger than in pHi. In the rats intraperitoneally administered with 0.5 ml/kg of bromobenzene, pHe and pHi were 7.412 ± 0.003 and 7.137 ± 0.013 (mean \pm S.E., $n=5$) at 24 hr after injection, and 7.395 ± 0.008 and 7.129 ± 0.014 ($n=3$) at 48 hr, respectively. These values showed no significant difference from those of the controls. All rats treated with 1.0 ml/kg died within 24 hr after injection.

Discussion

Simultaneous administration of PB or 3MC inhibits azo-dye induced hepatocarcinogenesis. This inhibition is thought to stimulate the activation of microsomal drug metabolizing enzymes in the liver such as N-demethylase and azo reductase, which subsequently transform into non-carcinogenic metabolites.⁴⁾ We found that the treatment of rats with PB or 3MC increased azo reductase activity for more than one week. Conney *et al.*¹²⁾ reported that a single intraperitoneal injection of 1.0 mg of 3MC effected a maximum increase in azo reductase activity at 3 days which returned to that of the controls at approximately 6 days, and that the administration of large amounts of 3MC effected a greater increase in enzyme activity which persisted for a longer period before returning to the normal level. Orrenius *et al.*¹³⁾ reported that after cessation of PB treatment, oxidative N-demethylase and NADPH-cyt.c reductase activities and the amount of CO-binding pigment were increased and that they returned to their original levels within approximately 5 days.

The pHi decrease observed earlier in rats on a 3'-Me-DAB diet³⁾ may be useful in elucidating the action of this carcinogen. In the present investigation, PB or 3MC pretreatment prevented pHe and pHi decreases in rats on a 3'-Me-DAB diet. We do not think that the prevention of the pHi decrease is due to acceleration of 3'-Me-DAB metabolism, but rather, that it is due to the elimination or reduction of the hepatotoxicity of 3'-Me-DAB. We noted earlier that 3'-Me-DAB treatment induced marked cell proliferation in rat livers and it is of interest to discover whether increased cell proliferation is related to the decrease in pHi. Partially hepatectomized rats on the basal diet maintained pHe and pHi levels within the normal range and rats fed 3'-Me-DAB for 5 days evidenced significant decreases in pHi, while pHe remained within normal limits. A tendency of decreased pHi and normal pHe was also noted in non-hepatectomized rats fed the 3'-Me-DAB diet for 7 days.³⁾ In partially hepatectomized rats, administration of 3'-Me-DAB may induce a greater decrease in pHi than in non-hepatectomized rats. Warwick reported that the administration of 2-Me-DAB to partially hepatectomized rats effected hepatocarcinogenesis¹⁴⁾ and we now posit that 2-Me-DAB decreases pHi in the regenerating liver, although the administration of 2-Me-DAB to normal rats did not result in a pHi decrease.³⁾ Therefore, we investigated whether the pHi decrease in normal rats on a 3'-Me-DAB diet could be induced by other hepatocarcinogens. Rubin *et al.*¹⁵⁾ reported that at concentration of 50 ppm, DENA caused little cholangiofibrosis and that

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the DENA induced liver injury was different from that induced by 3'-Me-DAB. The pHe and pHi decrease induced by DENA was smaller than that induced by 3'-Me-DAB, suggesting that these drugs affect different liver sites.

We administered CCl₄ and bromobenzene which are known to possess different modes of toxic action and found that their effect on pHi was smaller than that of 3'-Me-DAB. The CCl₄ experiments revealed a correlation between dosage and response, *i.e.* in rats administered with less than 2.0 ml/kg, pH values after 48 hr were closer to control levels than after 24 hr, indicating that the drug was nearly completely metabolized to inactive at 48 hr. This finding further indicates that some mechanism(s) which returned pH the normal level was operative following the injection of CCl₄. In rats administered with CCl₄ in excess of 2.0 ml/kg, pH levels were higher at 24 than at 48 hr, possibly because the rate of repair is inhibited by the greater dosage amount, thereby allowing more time for the action of the drug. Five ml CCl₄ represents the LD₉₅ at 48 hr. Ugazio *et al.*¹⁶⁾ reported that in rats, below-lethal doses of CCl₄ resulted in maximal or nearly maximal increases in liver weight and liver neutral lipid content during the first 24—48 hr after intoxication.

We observed the pHe and pHi changes induced by compounds with different toxic mechanisms, *i.e.* 3'-Me-DAB, 2-Me-DAB, DENA, CCl₄ and bromobenzene, and noted that their effects were different. Whether the difference in drug effect is due to the difference in toxic mechanisms or drug metabolism remains to be discovered. We further found that there is no direct correlation between extent of liver damage and degree of pH decrease. However, we suggest that a decrease in liver pH may reflect the occurrence of liver damage, based on the observations that administration of 2-Me-DAB did not effect a decrease in pH and that pretreatment with PB or 3MC prevented the 3'-Me-DAB induced pH decrease.

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