Short Communications

Effects of Adrenalectomy and Bile-Duct Ligation on the Urinary Excretion of Metabolites of 2-Acetamidofluorene by Male Weanling Rats

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The versatile carcinogen AAF* is metabolized by the rat mostly to inactive ring-hydroxylated phenolic metabolites, which are excreted in the urine primarily as glucuronides and sulphates (Weisburger, Weisburger & Morris, 1956a; Weisburger, Weisburger, Morris & Sober, 1956b; Miller, Cramer & Miller, 1960). *N*-Hydroxy-AAF was also shown to be excreted in the rat urine in the form of *N*-hydroxy-AAF *O*-glucuronide (Cramer, Miller & Miller, 1960b). It was subsequently demonstrated that *N*-hydroxy-AAF was more carcinogenic than the parent amide not only for the rat but also for other rodent species (Miller, Miller & Hartmann, 1961; Miller, Enomoto & Miller, 1962; Miller, Miller & Enomoto, 1964).

Incidence of liver tumour formation by 2-amino-fluorene and its acetylated derivatives AAF and *N*-acetamido-AAF was shown to be much lower in adrenalectomized than in control male rats (Firminger & Reuber, 1961; Perry, 1961; Reuber & Firminger, 1962; Reuber, 1969). Administration of cortisone restored the carcinogenicity in these animals (Firminger & Reuber, 1961; Reuber, 1969).

In previous studies Lotlikar, Enomoto, Miller & Miller (1964) reported that adrenalectomized young male rats after a test dose of either *N*-acetamido-AAF or AAF excreted in their urine only about 40% as much *N*-hydroxy-AAF as unoperated controls whereas administration of cortisone to these adrenalectomized rats restored the urinary *N*-hydroxy-AAF excretion almost to control values. It was suggested then that one of the roles of adrenal hormones in promoting hepatic carcinogenesis by AAF and *N*-acetamido-AAF in the rat may be to promote the formation or maintenance of higher concentrations of the *N*-hydroxy derivative. Irving, Wiseman & Hill (1967) have reported that only a small fraction of the *N*-hydroxy-AAF formed by the rat was excreted in the urine as *N*-hydroxy-AAF *O*-glucuronide whereas a large fraction was excreted in the bile. They also demonstrated that the *N*-hydroxy-AAF *O*-glucuronide

usually excreted primarily in the bile of normal rats could be shunted to the urine by ligation of the common bile duct.

The present studies were undertaken primarily to re-examine the effects of adrenalectomy on the urinary excretion of *N*-hydroxy-AAF after administration of a test dose of AAF to young male rats whose bile ducts had been ligated.

Twentyfour male weanling rats (50-60g body wt.) of the Sprague-Dawley strain were obtained from Holtzman Rat Co., Madison, Wis., U.S.A. One group of eight rats was adrenalectomized in our laboratory. All rats were given Rockland rat/mouse diet and water *ad libitum*. Adrenalectomized rats were given 1% sodium chloride in their drinking water. At 12 days after adrenalectomy only five out of eight rats had survived. On that day the common bile duct of each of the adrenalectomized group and of another group of eight rats was ligated according to the procedure of Cameron & Oakley (1932). At 4-5h after bile-duct ligation these animals and the controls were injected intraperitoneally with AAF (Mann Research Laboratories, New York, N.Y., U.S.A.) suspended in a 1.75% gum acacia and 0.9% sodium chloride solution (3mg of AAF/100g body wt.). All animals were housed in individual metal cages; 24h-urine samples were collected under solid CO₂. Only drinking water was available to these animals during urine collection.

Urine samples were thawed, filtered through glass wool and diluted to 60ml after adjustment of the pH to 6.0. Two 20ml portions of the urine samples were incubated with Takadiastase (Parke, Davis and Co., Detroit, Mich., U.S.A.) and β -glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) as described previously (Miller *et al.* 1960; Weisburger *et al.* 1956b). After incubation for 18h at 37°C hydrolysed fluorene derivatives were extracted with diethyl ether. The ether extract was washed successively with 0.5M-hydrochloric acid and water. Acidic metabolites were chromatographed on Whatman no. 1 paper with the solvent system cyclohexane-2-methylpropan-2-ol-acetic

* Abbreviation: AAF, 2-acetamidofluorene.

Table 1. *Effects of adrenalectomy and bile-duct ligation on the urinary excretion of metabolites of AAF by young male rats*

Duplicate portions of each rat urine were taken for analyses. All other details are described in the text. The amounts of 1-hydroxy-AAF were too low to measure adequately and the values of this metabolite have therefore been omitted. Results are given as means $\pm$ S.E.M. * Statistical comparisons ($P < 0.025$) between control and ligated groups; † statistical comparisons ($P < 0.025$) between ligated group and adrenalectomized-ligated group. Statistical comparisons with P values < 0.025 are considered highly significant. All other statistical comparisons had P values > 0.20 and were considered not significant.

Group	No. of rats used	Body wt. when injected with AAF (g)	Urinary excretion of metabolites (% of dose)				
			N-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF	AAF
Control	7	144 $\pm$ 10	1.55 $\pm$ 0.59	2.04 $\pm$ 0.59	4.81 $\pm$ 0.39	11.7 $\pm$ 2.25	0.17 $\pm$ 0.14
Ligated	8	151 $\pm$ 9	3.52 $\pm$ 0.74*	4.16 $\pm$ 1.75*	6.80 $\pm$ 1.27*	18.0 $\pm$ 3.27*	0.09 $\pm$ 0.08
Adrenalectomized-ligated	5	130 $\pm$ 14	2.03 $\pm$ 0.87†	4.52 $\pm$ 0.79	6.44 $\pm$ 2.12	15.4 $\pm$ 4.82	0.15 $\pm$ 0.13

acid-water (16:4:2:1, by vol.) (Weisburger *et al.* 1956b). After chromatography appropriate u.v.-absorption zones were cut out and eluted overnight in 3 ml of ethanol. Before spectral analysis the *N*-hydroxy-AAF was separated from AAF by extraction with 0.5M-sodium hydroxide. The metabolites were determined quantitatively by u.v. absorption in the range 350–270 nm by using a Hitachi-Coleman recording spectrophotometer. The molar extinction coefficient and percentage recovery for these metabolites were used as described previously (Lotlikar, 1970).

Administration of a test dose of AAF 4–5 h after bile-duct ligation of control animals caused more than twofold increase in the urinary excretion of *N*-hydroxy-AAF compared with unoperated controls (Table 1). Likewise the excretion of 3-, 5- and 7-hydroxy-AAF was also increased to an appreciable extent in the operated group compared with unoperated controls. However, bile-duct ligation of adrenalectomized male rats showed a urinary excretion pattern different from that of the ligated group. A striking and significant difference between the two groups was the extent of *N*-hydroxy-AAF excretion. Thus *N*-hydroxy-AAF excretion in the adrenalectomized-ligated group was decreased to about 58% of that in the ligated group. In these groups excretion of 3-, 5- and 7-hydroxy-AAF was not significantly affected.

Like those reported by Irving *et al.* (1967), the results presented in this paper indicate that, when rats were injected with AAF a few hours after bile-duct ligation, the urinary excretion of *N*-hydroxy-AAF was increased to an appreciable extent compared with the unoperated controls. Irving *et al.* (1967) reported the presence of 3- and 5-hydroxy-AAF as glucuronides and 7-hydroxy-AAF both as glucuronides and sulphate in the rat bile after a test dose of AAF, but they did not report the urinary

excretion of these metabolites after bile-duct ligation. In our present studies the urinary excretion of ring-hydroxy metabolites, like that of *N*-hydroxy-AAF, was also increased to an appreciable extent after bile-duct ligation.

In the present experiments even after bile-duct ligation adrenalectomized male rats showed significant decrease in the urinary excretion of *N*-hydroxy-AAF compared with the ligated group. Adrenalectomy did not affect the excretion of ring-hydroxy metabolites. These results are in agreement with previous studies (Lotlikar *et al.* 1964) that were carried out without bile-duct ligation. In a separate experiment (P. D. Lotlikar & M. Gruenstein, unpublished work) it was observed that cortisone administration to adrenalectomized rats restored the urinary excretion of *N*-hydroxy-AAF to control values. In these studies AAF was administered 4 h after bile-duct ligation to both groups of rats.

It has already been demonstrated that urinary excretion of *N*-hydroxy-AAF was not affected by adrenalectomy when *N*-hydroxy-AAF was administered to young male rats (Lotlikar *et al.* 1964). Reduction of *N*-hydroxy-AAF to AAF by livers from adrenalectomized rats was also not different from that by control rat livers (Lotlikar *et al.* 1964). DeBaun, Miller & Miller (1970) have demonstrated that adrenalectomy, with or without castration, of adult male rats does not alter the *N*-hydroxy-AAF sulphotransferase activity of livers. These results indicate that adrenalectomy does not affect the breakdown or further metabolism of *N*-hydroxy-AAF. Therefore it appears that adrenal hormones regulate the formation of *N*-hydroxy-AAF. AAF has been shown to be ring-hydroxylated (Booth & Boyland, 1957; Cramer, Miller & Miller, 1960a; Seal & Gutmann, 1959) and *N*-hydroxylated (Irving, 1964; Lotlikar, Enomoto, Miller & Miller,

1967) by rat liver microsomes in the presence of NADPH and molecular oxygen. But amounts of *N*-hydroxy-AAF formation *in vitro* are very low and variable (Irving, 1964; Lotlikar *et al.* 1967; Lotlikar, 1970). Therefore it is very difficult at the present time to test the effects of adrenalectomy on *N*-hydroxylation of AAF *in vitro*.

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