Effects of Sex Hormones on Enzymic Esterification of 2-(*N*-Hydroxyacetamido)fluorene by Rat Liver Cytosol

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1. Enzymic esterifications of 2-(*N*-hydroxyacetamido)fluorene and several other hydroxamic acids by liver cytosol were studied. Determination of 2-acetamido-3-methylthiofluorene was used for the assay. 2. With rat liver cytosol, requirement for ATP, Mg^{2+} and SO_4^{2-} suggested formation of phosphate and sulphate esters of 2-(*N*-hydroxyacetamido)fluorene. 3. Rats showed sex and age differences in their activity. Liver from adult male rat was at least twice as active as liver from adult female rat. No such sex differences were found in mice, hamsters and guinea pigs. 4. Administration of testosterone (300 $\mu g/day$) subcutaneously for 8 days increased the activity in the female rat by 100%, whereas diethylstilboestrol (100 $\mu g/day$) had no effect. In the male rat diethylstilboestrol treatment decreased the activity by 60%, whereas testosterone pretreatment was without any effect. 5. Among various endocrine ablations such as adrenalectomy, castration, adrenalectomy-castration and hypophysectomy in the adult male rat, hypophysectomy was found to be the most effective in decreasing the activity of the liver to about 50% of control values. 6. Like 2-(*N*-hydroxyacetamido)fluorene, various other *N*-hydroxy derivatives of 2-acetamido-7-fluorofluorene, 2-acetamidophenanthrene, 4-acetamidobiphenyl and 4-acetamidostilbene were also shown to be esterified to different extents by rat liver cytosol.

Several hormones have been shown to be involved in the formation of liver tumour in the rat by several aromatic amines. Thus many investigators have demonstrated a lower incidence of hepatoma induction by amino azo-dyes, 2-amino-fluorene and its acetylated derivatives, AAF* and 2-diacetamidofluorene in adrenalectomized (Eversole, 1957; Firminger & Reuber, 1961; Griffin, Richardson, Robertson, O'Neal & Spain, 1955; Perry, 1961; Reuber, 1969; Reuber & Firminger, 1962; Symeonidis, Mulay & Burgoyne, 1954), hypophysectomized (Bielschowsky, Bielschowsky & Fletcher, 1962; Dodge, O'Neal, Chang & Griffin, 1961; Griffin, Rinfret & Corsiglia, 1953; O'Neal, Hoffman, Dodge & Griffin, 1958), thyroidectomized (Bielschowsky & Hall, 1953; Bielschowsky, 1958) or castrated (Firminger & Reuber, 1961; Morris & Firminger, 1956; Reuber, 1969; Reuber & Firminger, 1962) male rats. Administration of cortisone, pituitary hormones (adrenocorticotrophin and growth hormone), thyroxine and male sex hormones restored the carcinogenicity in these animals (Bielschowsky, 1958; Bielschowsky *et al.* 1962; Dodge *et al.* 1961; Eversole, 1957; Firminger & Reuber, 1961; Reuber, 1969). In several strains of rats the male is shown to be more susceptible

than the female to hepatoma induction by many aromatic amines (Bielschowsky, 1944; Firminger & Reuber, 1961; Kirby, 1947; Leathem, 1951; Miller, Miller, Sandin & Brown, 1949; Morris & Firminger, 1956; Reuber, 1969; Symeonidis, 1954; Stasney, Paschkis, Cantarow & Rothenberg, 1947). Tumour incidence in the male rat was decreased by diethylstilboestrol whereas tumour incidence was increased in the female by administration of male sex hormones (Firminger & Reuber, 1961; Morris & Firminger, 1956; Reuber, 1969).

Since the first report of *N*-hydroxylation of AAF by Cramer, Miller & Miller (1960b) it has been demonstrated that *N*-hydroxylation is the first step of activation not only of AAF but also of several other aromatic amines and amides. Thus *N*-hydroxy derivatives are more carcinogenic than their corresponding amines and amides (Miller, Miller & Hartmann, 1961a; Boyland, Dukes & Grover, 1963; Miller, Wyatt, Miller & Hartmann, 1961b; Andersen, Enomoto, Miller & Miller, 1964; Baldwin, Smith & Surtees, 1963; Miller, Lotlikar, Pitot, Fletcher & Miller, 1966). The potent carcinogen AAF is metabolized by rat liver microsomes not only to various non-carcinogenic ring-hydroxylated products (Booth & Boyland, 1957; Seal & Gutmann, 1959; Cramer, Miller & Miller, 1960a) but also to

* Abbreviation: AAF, 2-acetamidofluorene.

more carcinogenic *N*-hydroxy derivatives (Irving, 1964; Lotlikar, Enomoto, Miller & Miller, 1967a). The effects of endocrine ablations on the metabolites of AAF in urine in the rat suggested that one of the roles of adrenal hormones in promoting hepatic carcinogenesis by AAF and 2-diacetamidofluorene in the rat might be to promote the formation and maintenance of higher concentrations of the *N*-hydroxy derivative (Lotlikar, Enomoto, Miller & Miller, 1964).

It has been shown that the synthetic *N*-acetoxy-AAF and several other *N*-acetoxy amides are more carcinogenic than their corresponding hydroxamic acids (Miller & Miller, 1969a; Miller, 1970). Thus synthetic *N*-acetoxy-AAF, AAF *N*-sulphate and AAF *N*-phosphate react with proteins, nucleic acids and their constituents non-enzymically (Lotlikar, Scribner, Miller & Miller, 1966; Kriek, Miller, Juhl & Miller, 1967; Lotlikar, Irving, Miller & Miller, 1967b; Fink, Nishimura & Weinstein, 1970; Miller & Miller, 1969b), whereas *N*-hydroxy-AAF does not react with these molecules (Bahl & Gutmann, 1964; King, Gutmann & Chang, 1963; Miller, Juhl & Miller, 1966; Miller *et al.* 1968). On the basis of these and other studies it was suggested that esterification of the *N*-hydroxy amides might be the second and final step of activation of the carcinogenic amines and amides (Miller & Miller, 1969b; Miller, 1970).

Rat liver is capable of conjugating many of the foreign chemicals as acetates, sulphates and glucuronides (Williams, 1967). King & Phillips (1968) first demonstrated that *N*-hydroxy-AAF could be activated enzymically by rat liver cytosol to reactive esters, presumably AAF *N*-phosphate and AAF *N*-sulphate. Liver from the male rat forms severalfold more AAF *N*-sulphate than that from the female rat (DeBaun, Rowley, Miller & Miller, 1968). These authors also showed that livers from mice, rabbit, hamsters and guinea pigs formed much smaller amounts of AAF *N*-sulphate than liver from the rat.

The present investigations were undertaken to determine whether sex differences in the rat observed with their liver sulphotransferase activity could be seen with other species and whether these differences could be modified by pretreatment with sex hormones. It was also important to investigate whether endocrine ablations in the male rat could also cause any changes in the enzymic esterification of *N*-hydroxy-AAF by the liver. Results of some of these studies were published in a preliminary report (Lotlikar, 1969).

MATERIALS AND METHODS

Animals. In all experiments Sprague-Dawley-strain male and female rats of various age and weight groups were used. These and hypophysectomized male rats

(150g body wt.) were obtained from Charles River Breeding Laboratories, Wilmington, Mass., U.S.A. Castration and adrenalectomy were performed in my laboratory. Adrenalectomized and adrenalectomized-castrated rats were given 1% NaCl and hypophysectomized rats 5% glucose in their drinking water. Adult male and female Swiss mice (20–25g body wt.) were obtained from Carworth Farms Co., New City, N.Y., U.S.A. Adult Syrian golden hamster (100–110g body wt.) and guinea pigs (350–400g body wt.) were purchased from Huntington Farms Inc., West Conshohocken, Pa., U.S.A. All animals were fed on commercial diets.

Chemicals. *N*-Hydroxy-AAF was prepared as described by Poirier, Miller & Miller (1963). The following synthetic compounds were kindly supplied by Dr J. A. Miller of the McArdle Laboratory, University of Wisconsin, Madison, Wis., U.S.A.: 7-fluoro-*N*-hydroxy-AAF, 2-(*N*-hydroxyacetamido)phenanthrene (Miller *et al.* 1966), 4-(*N*-hydroxyacetamido)stilbene (Andersen *et al.* 1964), 4-(*N*-hydroxyacetamido)biphenyl (Miller *et al.* 1961b), 3-methylthio-AAF and 2-amino-3-methylthiofluorene. L-[³⁵S]Methionine (25–30mCi/mmol) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Diethylstilboestrol (U.S.P. grade) was a product of Merck and Co. Inc., Rahway, N.J., U.S.A. Testosterone propionate (100mg/ml of sesame oil) was purchased from United Research Laboratories Inc., Philadelphia, Pa., U.S.A. ATP was a product of Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of reagent grade.

Injection of hormones. Diethylstilboestrol or testosterone propionate were suspended in sesame oil. Animals were injected subcutaneously with hormone suspended in 0.2ml of sesame oil. Control animals were injected with sesame oil.

Preparation of liver cytosol. After the animals were decapitated, the livers were immediately removed and chilled in ice-cold 0.25M-sucrose solution. After livers were blotted dry with filter paper, their total weights were recorded. A 20% (w/v) liver homogenate prepared in 0.1M-tris-HCl buffer, pH 7.0, was centrifuged at 105 000g for 60 min. The supernatant was used for enzyme assay.

Assay for enzymic esterification of *N*-hydroxy-AAF and other hydroxamic acids. Since synthetic esters of *N*-hydroxy-AAF, AAF *N*-sulphate and AAF *N*-phosphate are very reactive and have very short half-lives (Lotlikar *et al.* 1967b; Maher, Miller, Miller & Szybalski, 1968) it was not possible to isolate such reactive esters from incubation mixtures *in vitro*. Therefore the non-enzymic reaction of these esters with methionine to form 3-methylthio-AAF has been used as an assay system for enzymic esterification of *N*-hydroxy-AAF. Formation of 2-amino-3-methylthiofluorene is mostly due to enzymic deacetylation of *N*-hydroxy-AAF to form 2-hydroxyaminofluorene, which then reacted directly with methionine (King & Phillips, 1968). Some amounts of 2-amino-3-methylthiofluorene are also derived from enzymic deacetylation of 3-methylthio-AAF.

Unless otherwise indicated the complete incubation mixture contained: tris-HCl buffer, pH 7.0, 25mM; ATP, pH 7.0, 10mM; MgCl₂, 5mM; Na₂SO₄, 10mM; L-methionine containing [³⁵S]methionine (10⁶d.p.m.), 20mM; *N*-hydroxy-AAF, 1mM; dimethyl sulphoxide used for dissolving *N*-hydroxy-AAF, 705mM; 105 000g super-

natant, equivalent to 100mg wet wt. of liver; water, to a final volume of 2.0ml. After incubation in air for 2h at 37°C, 0.25ml each of ethanol and 8M-KOH were added and *o*-methyl[³⁵S]thio amide and *o*-methyl[³⁵S]thio amine were extracted in benzene. Benzene extract washed several times with water was dried under N₂; the residue was dissolved in benzene and chromatographed on silicic acid paper with solvent system benzene-chloroform (2:3, v/v). Chromatography was terminated after the solvent front had travelled 15cm from the origin. Then 1cm zones were cut, put into vials and eluted in 1ml of methanol for 30min; 10ml of Bray's (1960) solution was added to each vial and radioactivity was measured in a Packard Tri-Carb spectrometer. Radioactivity zones corresponding to synthetic 3-methylthio-AAF (*R_F* 0.35–0.45) and 2-amino-3-methylthiofluorene (*R_F* 0.67–0.77) were used for calculation of amounts of these two products formed. Values for 2h-incubated samples were corrected by subtracting the value for zero-time samples. Wherever values of 5 or less nmol/2h per 100mg wet wt. of liver are given for 3-methylthio-AAF this indicates that a small amount of radioactivity is being eluted that is slightly above that of zero-time sample. When other hydroxamic acids were used as substrates, benzene extracts on silicic acid paper chromatography with solvent system described above gave two distinct radioactivity peaks with *R_F* values similar to those of synthetic 3-methylthio-AAF and 2-amino-3-methylthiofluorene. These zones were used for calculations of amounts of the respective *o*-methylthio amide and *o*-methylthio amine formed.

Quantitative determination of 3-methylthio-AAF was also made by means of g.l.c. A Glowall model 10 chromatograph with a hydrogen flame detector was employed. A 1.8m circular glass column of 4mm int. diam. was packed with Gas-Chrom Q (80–100 mesh) coated with 1% SE-30 (supplied by Applied Science Laboratories Inc., State College, Pa., U.S.A.). Detector, column and flash-heater temperatures were maintained at 240°C, 210°C and 250°C respectively. With an argon flow rate of 45 ml/min, voltage

was kept at 300V. The residue of benzene extract from incubation sample was dissolved in 50–100μl of glass-distilled ethyl acetate. As a routine 1–10μl of ethyl acetate extract was injected for the determination. Synthetic 3-methylthio-AAF had a retention time of about 7.5min. About 0.2nmol of 3-methylthio-AAF present in 10μl of ethyl acetate could be easily determined. There was very close agreement between these two methods of analysis (g.l.c. and paper chromatography followed by radioactivity measurements) when extracts from rat liver incubation samples were tested for their 3-methylthio-AAF contents. However, benzene extracts of liver samples from mice, hamsters and guinea pigs were assayed for their 3-methylthio-AAF and 2-amino-3-methylthio-fluorene contents by paper chromatography followed by radioactivity measurements only.

RESULTS

Preliminary studies with a complete incubation medium i.e. ATP, Mg²⁺, SO₄²⁻, methionine, *N*-hydroxy-AAF and adult male rat liver 105000g supernatant, indicated that, with the equivalent of 25mg wet wt. of liver, formation of 3-methylthio-AAF was linear with time when studied over a period of 2h. With supernatant equivalent to 50 and 100mg wet wt. of liver there was a lag period for the first 30min, but then the rate was almost linear up to 2h. In all the studies reported here supernatant equivalent to 100mg wet wt. of liver was incubated for 2h.

The formation of 3-methylthio-AAF was dependent on several cofactors (Table 1). In the absence of ATP, Mg²⁺ and SO₄²⁻ or with ATP alone very little product was formed. In the presence of ATP and Mg²⁺ appreciable amounts of 3-methylthio-AAF could be detected. Similar results have also been obtained by other investigators (King &

Table 1. *Effects of various cofactors on enzymic activation of N-hydroxy-AAF by rat liver cytosol*

Two adult male rats weighing 280–300g were used in this study. Liver was homogenized in 0.1 M-tris-HCl buffer, pH 7.0. The supernatant obtained after 105000g centrifugation for 60min was used for enzyme assay. The incubation medium contained 50μmol of tris-HCl buffer, pH 7.0, 40μmol of L-methionine, 2μmol of *N*-hydroxy-AAF dissolved in 0.1 ml of dimethyl sulphoxide, rat liver supernatant equivalent to 100mg wet wt. of liver, other cofactors as indicated below and water to a volume of 2.0ml. + and ++++ represent 5mM and 20mM respectively of final Mg²⁺ concentrations. Assay of 3-methylthio-AAF was made by g.l.c.

Addition of cofactors				3-Methylthio-AAF (nmol formed/2h per 100mg wet wt. of liver)
ATP (10mM)	Mg ²⁺	SO ₄ ²⁻ (10mM)	Mn ²⁺ (5mM)	
—	—	—	—	<1
+	—	—	—	<1
+	+	—	—	35
+	+	+	—	133
+	+	+	—	<1 (0h)
+	++++	+	—	14
+	—	+	—	20
+	—	+	+	96

Phillips, 1968; DeBaun *et al.* 1968), who suggested the involvement of a phosphokinase that esterified *N*-hydroxy-AAF to AAF *N*-phosphate. Addition of SO_4^{2-} with ATP and 5mm- Mg^{2+} increased the product formation by about fourfold. These results are also in agreement with those reported by King & Phillips (1968) and DeBaun *et al.* (1968). They suggested the involvement of sulphotransferase, i.e., sulphate from adenosine 3'-phosphate 5'-sulphatophosphate that is formed by liver enzymes in the presence of ATP, Mg^{2+} and SO_4^{2-} is transferred enzymically to *N*-hydroxy-AAF to form AAF *N*-sulphate. Both of these esters, AAF *N*-phosphate and AAF *N*-sulphate, then react non-enzymically with methionine to form 3-methylthio-AAF. In these studies excess of Mg^{2+} (20mm) inhibited the reaction to an appreciable extent. According to King & Phillips (1968) Mn^{2+} could substitute for Mg^{2+} in the sulphotransferase reaction almost completely. The present results also show a similar effect.

In a separate experiment rat liver supernatant that had been dialysed against 100 vol. of cold deionized water with three changes of water during 2h was compared with the non-dialysed liver supernatant for cofactor requirements, especially

for ATP and Mg^{2+} ; they both showed similar activity. These results exclude the possibility of the presence of free SO_4^{2-} in the dialysed liver supernatant.

When livers from several adult species were tested for their sex differences a significant difference was found between male and female rats: this was about twofold, both in 3-methylthio-AAF and 2-amino-3-methylthiofluorene formation (Table 2). DeBaun *et al.* (1968) have also reported severalfold differences between male and female rats. In the present experiments there were not significant sex differences in mice, hamsters or guinea pigs.

Esterification of *N*-hydroxy-AAF by rat liver was also dependent on age (Table 3). Thus liver from 2-week-old male and female rats had low activity compared with that from adult male rats. The activity in the female reached a value 50% of that of the adult male at 4–5 weeks of age and remained at that value thereafter. However, activity in the male increased gradually.

It was important to investigate whether differences in enzyme activities observed between adult male and female rats could be modified by treatment with sex-hormones. Results obtained after 8 days of pretreatment with sex hormones are presented in Table 4. Body weight and liver

Table 2. *Species and sex differences in activation of N-hydroxy-AAF by liver*

All details are described in the Materials and Methods section. Results are given as means $\pm$ S.E.M.

Species	Sex	No. of animals (and analyses)	Amount formed (nmol/2h per 100mg wet wt. of liver)	
			3-Methylthio-AAF	2-Amino-3-methylthiofluorene
Rat	M	53 (45)	103 $\pm$ 29	17 $\pm$ 4.6
	F	22 (17)	45 $\pm$ 27	8.4 $\pm$ 5.8
Mouse	M	35 (8)	2.8 $\pm$ 1.9	8.0 $\pm$ 4.5
	F	26 (5)	6.2 $\pm$ 1.2	4.0 $\pm$ 1.5
Hamster	M	8 (5)	19 $\pm$ 0.4	9 $\pm$ 2.3
	F	6 (3)	20 $\pm$ 1.9	9 $\pm$ 0.8
Guinea pig	M	5 (5)	11 $\pm$ 4	7 $\pm$ 2.4
	F	2 (2)	10 $\pm$ 1	7 $\pm$ 1

Table 3. *Age and sex differences in esterification of N-hydroxy-AAF by rat liver*

Livers from two or three animals were used per analysis. In these studies 2-week-old rats were not separated into males and females for enzyme assay. All other details are described in the Materials and Methods section. 3-Methylthio-AAF formation is expressed as percentages of that formed by adult male rats weighing about 200g. Results are averages of three analyses.

Animals			3-Methylthio-AAF formation (% of that formed by adult male rat liver)
Age (weeks)	Body wt. (g)	Sex	
2	17–18	M and F	25
4–5	50–70	M or F	50
7	120	F	50
7	150	M	80
10	200	F	50

Table 4. *Effects of pretreatment with diethylstilboestrol and testosterone on the esterification of N-hydroxy-AAF by livers from male and female rats*

Four adult rats (170–180 g) were used per group. Testosterone propionate (300 µg/day) and diethylstilboestrol (100 µg/day) suspended in sesame oil were injected subcutaneously per rat for 6 days. On days 7 and 8 the dose of these hormones was doubled. Control animals were injected with sesame oil alone. Animals were killed on day 9. At that time their body weights and liver wet weights were recorded. All other details are described in the Materials and Methods section. Results are given as means $\pm$ s.e.m. of four determinations.

Treatment	Sex	Body wt. (g)	Liver wet wt. (g)	Amount formed (nmol/2 h per 100 mg wet wt. of liver)	
				3-Methylthio-AAF	2-Amino-3-methylthiofluorene
Control	F	158 $\pm$ 6.2	8.0 $\pm$ 0.81	16 $\pm$ 3.4	4.4 $\pm$ 0.7
Diethylstilboestrol	F	112 $\pm$ 4.3	6.3 $\pm$ 0.43	13 $\pm$ 1.7	3.9 $\pm$ 0.4
Testosterone	F	165 $\pm$ 5.0	8.3 $\pm$ 0.81	33 $\pm$ 5.0	9.0 $\pm$ 2.0
Control	M	188 $\pm$ 8.3	9.3 $\pm$ 0.43	70 $\pm$ 4.3	16 $\pm$ 0.6
Diethylstilboestrol	M	138 $\pm$ 11.0	7.3 $\pm$ 0.82	27 $\pm$ 2.7	9.5 $\pm$ 1.5
Testosterone	M	203 $\pm$ 4.7	9.3 $\pm$ 0.47	64 $\pm$ 2.7	16 $\pm$ 1.2

Table 5. *Effects of pretreatment with sex hormones on the activation of N-hydroxy-AAF by livers from male and female rats*

Four adult rats (200–220 g) were used per group. Animals were injected subcutaneously with either testosterone propionate (10 mg) or diethylstilboestrol (1 mg) suspended in sesame oil 24 h before they were killed. Control animals were injected with sesame oil. All other details are described in the Materials and Methods section. Results are given as means $\pm$ s.e.m. of four determinations.

Treatment	Sex	Amount formed (nmol/2 h per 100 mg wet wt. of liver)	
		3-Methylthio-AAF	2-Amino-3-methylthiofluorene
Control	F	58 $\pm$ 2.1	12 $\pm$ 0.4
Diethylstilboestrol	F	43 $\pm$ 1.7	16 $\pm$ 4.7
Testosterone	F	83 $\pm$ 1.7	15 $\pm$ 2.4
Control	M	111 $\pm$ 1.6	20 $\pm$ 1.0
Diethylstilboestrol	M	123 $\pm$ 5.7	22 $\pm$ 1.3
Testosterone	M	113 $\pm$ 12	20 $\pm$ 2.6

wet weight were lower among diethylstilboestrol-treated male and female rats than in their respective control groups. In this study control males formed about fourfold as much 3-methylthio derivatives as did control females. Diethylstilboestrol pretreatment did not have any appreciable effect on the formation of reaction products in the female, whereas testosterone treatment doubled the activity compared with their controls. On the other hand testosterone had no appreciable effect on the male whereas diethylstilboestrol administration decreased 3-methylthio-AAF formation to about 40% of control values. In this treated group 2-amino-3-methylthiofluorene was also decreased to a large extent.

It was necessary to determine whether such changes in activity could be produced in adult rats in 24 h after pretreatment with a single large dose of these sex hormones. Pretreatment of male rats with a single large dose of either testosterone or

diethylstilboestrol did not have any appreciable effect on their product formations (Table 5). However, the formation of 3-methylthio-AAF by the female rat liver was decreased to about 75% of control values by the administration of 1 mg of diethylstilboestrol, whereas pretreatment with a large dose of testosterone increased the activity by about 40% of control values.

Since prolonged periods of treatment with sex hormone did affect the formation of 3-methylthio-AAF, it was decided to study the effect of various endocrine ablations including castration on the enzymic esterification of *N*-hydroxy-AAF. Results of such endocrine ablations in male rats are summarized in Table 6. The amount of 3-methylthio-AAF was not affected appreciably in rats that had been castrated 6 days before analysis; however, castration after a prolonged period (90 days) did decrease the product formation. Adrenalectomy alone appeared to cause an increase in 3-methylthio

Table 6. *Effects of endocrine ablations on esterification of N-hydroxy-AAF by rat liver*

Four to six adult male rats (180–190 g) were used per group. One group was castrated when animals were about 100 g body wt.; this castrated group was used for enzyme assay after 90 days. All other details are described in the Materials and Methods section. Results are expressed as percentages of the control calculated both on 100 mg wet wt. of liver and total wet wt. of liver. Results are averages of four analyses.

Group	Time after endocrine ablation (days)	3-Methylthio-AAF formed (% of control liver)		2-Amino-3-methylthio- fluorene formed (% of control liver)	
		(per 100 mg)	(total)	(per 100 mg)	(total)
Control		100	100	100	100
Adrenalectomized	7	118	63	124	65
Castrated	6	99	91	80	76
Castrated	90	60	52		
Adrenalectomized–castrated	6	97	72	123	93
Hypophysectomized	11	52	21	60	24

Table 7. *Activation of various aromatic hydroxamic acids by rat liver cytosol*

Two adult male rats weighing 260 g were used for this study. Incubation medium, except substrate, and other details are as described in the Materials and Methods section. The reaction products, *o*-methylthio amide and *o*-methylthio amine, were separated and determined quantitatively by paper chromatography and radioactivity measurements as described for the substrate *N*-hydroxy-AAF. Results are averages of duplicate analyses.

Substrate	Amount formed (nmol/2 h per 100 mg wet wt. of liver)	
	<i>o</i> -Methylthio amide	<i>o</i> -Methylthio amine
—	0	0.8
<i>N</i> -Hydroxy-AAF	80	12
7-Fluoro- <i>N</i> -hydroxy-AAF	65	11
4-(<i>N</i> -Hydroxyacetamido)stilbene	38	11
4-(<i>N</i> -Hydroxyacetamido)biphenyl	57	33
2-(<i>N</i> -Hydroxyacetamido)phenanthrene	11	22

derivatives when compared with controls on the basis of 100 mg of liver. On the basis of total liver, however, adrenalectomy showed a decrease of about 35%. Adrenalectomized–castrated rats also showed some inhibition of 3-methylthio-AAF formation when calculated on the basis of total liver. The striking decrease in activity was seen only with the hypophysectomized rats.

When various carcinogenic aromatic hydroxamic acids were tested for their enzymic esterification by rat liver cytosol, it was found that *N*-hydroxy-AAF was the most reactive substrate (Table 7). 2-(*N*-Hydroxyacetamido)phenanthrene was the least reactive substrate in the formation of *o*-methylthio amide derivative.

DISCUSSION

The results presented in this paper showing the requirements for ATP, Mg^{2+} and SO_4^{2-} in enzymic esterifications of *N*-hydroxy-AAF are similar to those reported by King & Phillips (1968) and DeBaun *et al.* (1968). These results suggested that

rat liver cytosol contained enzymes that esterified *N*-hydroxy-AAF to AAF *N*-phosphate and AAF *N*-sulphate. Because of the labile and reactive nature of these esters, they reacted non-enzymically with proteins, nucleic acids and their constituents, guanosine and methionine (King & Phillips, 1968, 1969; DeBaun *et al.* 1968; DeBaun, Miller & Miller, 1970). In the present studies these esters reacted with methionine to form 3-methylthio-AAF.

Among various adult species studied in this paper, male rat liver was found to be the most reactive in esterification of *N*-hydroxy-AAF. Sex differences were also observed in rats. Thus male rat liver was severalfold more active than that of the female in the formation of 3-methylthio-AAF. This sex difference in rats has also been shown with enzyme systems with generated or added adenosine 3'-phosphate 5'-sulphatophosphate (DeBaun *et al.* 1968). It therefore appears that the rate-limiting factor in those studies was not the adenosine 3'-phosphate 5'-sulphatophosphate-generating system but the sulphotransferase that converted *N*-hydroxy-AAF into AAF *N*-sulphate. Weisburger,

Grantham & Weisburger (1964) have also demonstrated that on administration of *N*-hydroxy-AAF the metabolites of *N*-hydroxy-AAF in the urine of male rats contained more sulphate conjugates and less of glucuronic acid conjugates than that of female rats. There is a possibility in the present experiments that, in addition to differences in liver sulphotransferase activity between male and female rats, there may be some differences in phosphokinase activity as well.

Many investigators have shown that, in several strains of rats studied so far, liver of male rats was more susceptible to AAF and 2-diacetamidofluorene carcinogenesis than that of female rats (Bielschowsky, 1944; Firminger & Reuber, 1961; Kirby, 1947; Leathem, 1951; Morris & Firminger, 1956; Reuber, 1969; Symeonidis, 1954). Likewise *N*-hydroxy-AAF was shown to be more carcinogenic to the liver of male rat than that of the female (Miller *et al.* 1961a; Weisburger, Yamamoto, Korzis & Weisburger, 1966). The results presented in this paper and those reported by DeBaun *et al.* (1968) could explain the carcinogenicity data on the basis of differences in enzyme activities involved in esterification of the hydroxamic acid.

Results presented here have also indicated that the enzyme activities in livers of male and female rats could be modified by administration of sex hormones for more than a week. Thus diethylstilboestrol treatment inhibited the formation of 3-methylthio-AAF by male rat liver whereas testosterone did not have any effect. On the other hand testosterone increased the activity in the liver of female rat whereas treatment with diethylstilboestrol was without any effect. When these sex hormones were given in a single large dose to male and female rats 24h before enzyme assay, these hormones did not show any appreciable effect on 3-methylthio-AAF formation. It was surprising to observe that a delayed response in male rats was also shown after castration. These results suggest that the sex hormones do not exert their primary effect on the liver. Kato, Chiesara & Frontino (1962) also observed such delayed effects of sex hormones on drug-metabolizing enzymes in liver. The effects of castration and sex hormones on hepatoma induction by acetylated aminofluorene derivatives in the rat (Firminger & Reuber, 1961; Morris & Firminger, 1956; Reuber, 1969) are in agreement with the present findings and those obtained by others (Miller, 1970; DeBaun *et al.* 1970).

Perry (1961) reported that adrenalectomy protected the livers of male rats from tumour induction by AAF. Reuber (1965) concluded that under his conditions both adrenalectomy and castration were necessary for inhibition of hepatic carcinogenesis by 2-diacetamidofluorene. Although Perry (1961) did not find castration as such required for in-

hibition of tumour in adrenalectomized rats, he did find testicular degeneration in those rats. As found by Reuber (1965), in the present experiments adrenalectomy alone was not sufficient to affect the esterification of *N*-hydroxy-AAF by male rat liver. Even castration in addition to adrenalectomy was without much effect. Whereas carcinogenicity studies are conducted over a period of several months, the present experiments were carried out only a week after adrenalectomy with or without castration. It might be possible that it takes a much longer time for esterification enzymes to be modified under certain hormonal imbalances. The present experiments with castrated animals support this possibility.

Hypophysectomy was the only endocrine ablation that showed a striking effect in the present studies. A similar effect was also observed by Miller (1970). In carcinogenicity studies Goodall (1968) demonstrated that hepatoma induction by *N*-hydroxy-AAF is inhibited to a large extent in hypophysectomized rats. Weisburger, Pai & Yamamoto (1964) found that rats bearing a transplanted pituitary tumour that secretes growth hormone, prolactin and adrenocorticotrophin were more susceptible to hepatic carcinogenesis by *N*-hydroxy-AAF than are normal rats.

The mechanisms by which various hormones influence hepatoma induction in rats by acetylated aminofluorenes and several other amines are not yet clearly understood. The results presented in this paper suggest that sex hormones might exert their secondary effect in hepatoma induction by regulating enzymes that are involved in esterification of *N*-hydroxy-AAF. Similarly hormones from pituitary, adrenals and thyroid may also have a similar role of modulating these enzymes.

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