Biochemical Pharmacology, Vol. 30, No. 24, pp. 3317–3322, 1981.
Printed in Great Britain.

0006-2952/81/243317-06 $02.00/0

STUDIES ON THE *IN VITRO* METABOLISM OF AFLATOXIN B_1 AND G_1 IN THE LIVER OF RATS FED AT VARIOUS LEVELS OF VITAMIN C

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(*Received* 5 *May* 1980; *accepted* 24 *June* 1981)

Abstract—Male and female weanling rats, divided into three groups, were maintained for 30, 60 and 90 days on an experimental diet without vitamin C. The drinking water containing or devoid of vitamin C was offered each day. The first group had no vitamin C; the second group or control had normal vitamin C and the third group had above-normal vitamin C in the water. Twenty-four hours after the last feeding, the animals were decapitated and the liver microsomes plus soluble fraction (9000 *g*) were prepared. This preparation was used for *O*-demethylase and the hydroxylase assays of aflatoxins B_1 and G_1 (AFB_1 and AFG_1). Results from *O*-demethylation in both control and experimental groups of both sexes, showed that *O*-demethylase activity is directly related to ascorbic acid content of the water. But the hydroxylase activity in male and female animals maintained on the same diet showed only slight differences. In the male, there was an increase with time of hydroxylase activity with AFB_1 up to 60 days and a decrease at 90 days. Hydroxylation assay revealed that in the female, metabolism of AFB_1 increased at 30 days but decreased at 60 and 90 days.

Several workers have shown that vitamin C deficiency results in decreased metabolism of many drugs [1–9]. These studies have demonstrated that *O*-demethylation, *N*-demethylation, hydroxylation reactions as well as individual liver microsomal electron transport components such as cytochrome P-450 and NADPH–cytochrome P-450 reductase are decreased in guinea pigs depleted of ascorbic acid. But when scorbutic guinea pigs are supplemented with ascorbic acid, cytochrome P-450 and demethylation activities returned to normal within 48 hr [10, 11]. Only very few investigations have considered the role of vitamins on the metabolism of mycotoxins. In this laboratory it has been shown, for example, that dietary vitamin A deficiency in the rabbit induces AFB_1 metabolism as judged by *O*-demethylation of this mycotoxin [12]. Despite the claimed importance of vitamin C in the etiology of cancer [13], no one has reported the role of vitamin C in the metabolism of mycotoxins. We therefore report the effect of vitamin C depletion and supplementation on AFB_1 and AFG_1 metabolism in rats.

MATERIALS AND METHODS

Treatment of animals

Male and female weanling Wistar albino rats were utilized. The animals were randomized and divided into three groups with equal numbers of males and females. Separate cages were used for each animal. All groups received the same basal diet *ad lib*. The first group received water without vitamin C. The second group (control) had 1.08 mg/ml vitamin C from the drinking water. The third group received 5.4 mg/ml similarly. Vitamin C solution was prepared freshly every day. The animals were maintained on the basal diet and the vitamin C dose for 30, 60 or 90 days respectively.

Microsomes plus supernatant (9000 g)

At the end of the experimental period, the animals were decapitated and their livers were quickly removed and kept in cold 0.9% NaCl (0–4°). The microsomes plus soluble fraction (9000 *g*) were prepared by the method previously described [14]. The livers of the rats of each dietary group were pooled according to sex to provide sufficient material for all subsequent assays. Livers were homogenized at 0–4° in 0.3 M phosphate buffer, pH 7.4, using a Waring blender. The homogenate was centrifuged at 9000 *g* for 30 min (4–6°) in a High Speed MSE centrifuge.

The supernatant containing the microsomal and soluble fractions of the liver cells was used in the *in vitro* studies and in protein and vitamin C estimations. All these estimations and assays were performed on the same day as the animals were killed.

Enzyme assays

Liver supernatant protein was determined by the biuret method [15] and the quantity of protein was expressed as mg/ml supernatant. Bovine Serum albumin (Koch–Light, Colnbrook, U.K.) was used as standard.

The enzyme assays were carried out in 25 ml Erlenmeyer flasks with Sigma reagents (Sigma Chemical Co., London, U.K.). The method previously described [16, 17] was slightly modified. Aliquots of the supernatant equivalent to 0.52 ± 0.13 g whole liver, were incubated with AFB_1 or AFG_1 (50 nmoles in DMSO) in a medium consisting of potassium phosphate buffer (0.1 M, pH 7.4), $NADP^+$ (0.52 μmoles), glucose-6-phosphate (50 μmoles), nicotinamide (50 μmoles). The total

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Table 1. Growth coefficients of rats on different levels of vitamin C

	I		II		III	
Weeks	Male	Female	Male	Female	Male	Female
0–6	0.31* ±0.05	0.32* ±0.01	0.50 ±0.021	0.25 ±0.01	0.50 ±0.013	0.32* ±0.02
0–12	0.23* ±0.05	0.00	0.16 ±0.08	0.00	0.23* ±0.021	0.00

Three groups of albino Wistar rats are fed with experimental diet without vitamin C. This latter is given in the drinking water.

I: Group with no vitamin C in the drinking water.

II: Group receiving 1.08 mg/ml vitamin C.

III: Group receiving 5.4 mg/ml vitamin C.

Animals are weighed weekly; the growth coefficient represents the slope of the growth curve.

Values represent mean ± S.E.M. of three experiments.

* Values which are significantly different from control group II at $P < 0.05$.

volume per flask was 5 ml. In separate experiments, the samples of AFB_1 and AFG_1 were shown to run as single spots on t.l.c. with silica gel 60 F-254 (Merck) in acetone–chloroform (10:90).

For the demethylase assay, semicarbazide (50 μmoles) was added. The incubation time was 1 hr at 37° in a Gallenkamp shaking incubator. The reaction was stopped by addition of 2 ml 35.6% zinc sulphate followed by 2 ml of saturated barium hydroxide solution. The precipitated protein was removed by centrifugation at 9000 *g* for 10 min. The supernatant was then decanted and used for formaldehyde estimation. The demethylation of aflatoxin B_1 and G_1 was determined using the Nash reagent [18] according to the method of Cochin and Axelrod [19] as modified by Stitzel *et al.* [20]. Specific activity was expressed as nmoles of HCHO produced/hr/mg of 9000 *g* supernatant protein at 37°.

The hydroxylase assay was terminated by the addition of a saturated solution of sodium chloride [21] followed by 5 ml of cold chloroform. The mixture was stored for no longer than 12 hr at 4° before being extracted 5 times with 10 ml of chloroform. The total extracts were then concentrated to 0.5–1 ml in the evaporator at 40° [22] prior to spotting on a t.l.c. plate. The determination of the quantity of aflatoxin B_1 metabolized was obtained from the method of Nabney and Nesbitt [23], and the formula of Rodrick and Stoloff [24]. The specific activity was expressed as nmoles of aflatoxin B_1 metabolized/hr/mg protein in the 9000 *g* supernatant.

Vitamin C in the liver was estimated by the method of Roe and Kuether [25]. Sigma ascorbic acid was used as standard.

RESULTS

Table 1 shows the growth coefficients for male and female rats. Rats with no vitamin C in the drinking water have values of 0.30 for male, and 0.32 for female between 0 and 6 weeks, showing that the growth curve is similar. But after 6 weeks or specifically between 6 and 11 weeks, the growth coefficient pattern is different, being higher in male than in female rats. Rats with 1.08 mg/ml of vitamin C in drinking water have growth coefficients of 0.50 for male and 0.25 for female, indicating that under that quantity of vitamin C intake, growth between 0–6 weeks is higher in male rats. After 6 weeks, however, the growth rate is reduced by three times in males with almost no variation in females. The growth coefficient in animals which receive 5.4 mg/ml vitamin C is similar to that of the group which has no vitamin C in the drinking water.

Table 2 shows the vitamin C content in the liver of rats after 30, 60, and 90 days of experimental

Table 2. Vitamin C content in the liver of rats fed various levels of vitamin C

Experimental groups	Vitamin C intake (mg/vitamin C/ml drinking water)	Days of feeding	Vitamin C in the liver (μg/g fresh liver)	
			Female	Male
I	0	30	30.02 ± 4.50	29.32 ± 2.32
		60	25.72 ± 3.72	28.82 ± 3.82
		90	29.50 ± 4.91	27.72 ± 0.91
II	1.08	30	45.03 ± 2.02	36.65 ± 1.91
		60	42.95 ± 0.53	35.95 ± 2.04
		90	38.64 ± 1.04	28.31 ± 2.14
III	5.4	30	50.12 ± 2.25	48.24 ± 1.25
		60	45.94 ± 5.20	39.90 ± 2.05
		90	53.52 ± 6.05	35.39 ± 1.93

Data represent mean ± S.E.M. of three experiments.

Table 3. Demethylation *in vitro* of aflatoxin B_1 and G_1 with livers of male and female rats fed at various levels of vitamin C/HCHO produced/hr/mg protein (nmoles)

	I		II		III	
AF	Male	Female	Male	Female	Male	Female
4 Weeks						
B_1	0.020 ± 0.007	0.007* ± 0.002	0.022 ± 0.006	0.048 ± 0.011	0.073* ± 0.021	0.007* ± 0.002
G_1	traces	0.007 ± 0.001	0.002 ± 0.001	0.080 ± 0.022	0.061* ± 0.011	0.049* ± 0.015
8 Weeks						
B_1	0.041 ± 0.013	0.016* ± 0.004	0.044 ± 0.003	0.056 ± 0.012	0.093* ± 0.019	traces
G_1	traces	0.101* ± 0.025	traces	0.086 ± 0.001	0.087 ± 0.022	0.091 ± 0.018
12 Weeks						
B_1	0.105* ± 0.028	0.072* ± 0.015	0.059 ± 0.011	0.220 ± 0.052	0.052 ± 0.013	0.097* ± 0.022
G_1	0.052* ± 0.012	0.123* ± 0.027	0.107 ± 0.031	0.157 ± 0.051	traces	0.042* ± 0.017

Livers were pooled for each dietary sub-group according to the sex. Five replicates of demethylases were made per assay.

Values represent mean ± S.E.M. of three experiments.

* Values which are significantly different from control (group II) at $P < 0.05$.

For description of groups I–III, see Table 1.

feeding. At 30 days as well as at 60 or 90 days, the amount of vitamin C in the liver of animals of both sexes increases with increased intake of the vitamin. For example at 60 days, compared to group I, the increase in group II is 67 per cent for females and 25 per cent for males; in group III it is 78 and 38 per cent respectively. However, in the same dietary group, females seem to concentrate more ascorbic acid consumed in the liver than males: in the group fed 1.08 mg vitamin C/ml, females' vitamin C content in the liver is 19 per cent higher than males'; and in the group fed 5.4 mg/ml it is 15 per cent higher.

Table 3 shows the formaldehyde produced by the 3 experimental groups of rats when their respective 9000 *g* hepatic supernatants were used to metabolize AFB_1 and AFG_1. These results (Table 3) are expressed as percentages on a comparative basis (Table 4).

AFB_1 metabolism as judged by *O*-demethylase activity at 4 weeks is 70 per cent greater with rats given 5.4 mg/ml vitamin C (group III) than those given 1.08 mg/ml vitamin C (group II), which do not differ from the group of male rats receiving no vitamin C (group I). However, with the female, the metabolism of aflatoxin B_1 showed considerable difference from the male: the formaldehyde production is 84 per cent greater in group II than in group I or group III. Aflatoxin B_1 metabolism in 12-week-old rats shows a difference as compared with those of 4 or 8 weeks. With males, the *O*-demethylase activity is 42 per cent higher in group I than in group II, whereas there is no difference between groups II and III. In the female group, the activity is 67 per cent higher in group II than in group I, and only 10 per cent higher than in group III. Intersex comparison of *O*-demethylase activity (Table 5), shows a difference between male and female. AFB_1 demethylase activities of male and female at 4 weeks indicate that in the group receiving no vitamin C, the *in vitro* production of formaldehyde is 59 per cent higher in the male animals than in the female. But in the group offered 1.08 mg/ml vitamin C, the demethylase activity is 54 per cent lower in the male than in the female. In the third group (5.4 mg/ml

Table 4. Comparative summary of Table 3

		Male					Female				
Weeks	Aflatoxins	I	–	II	–	III	I	–	II	–	III
1–4	AFB_1		ND		<	70%		<	84%	>	
	AFG_1		<*		ND			<	91%	ND	
5–8	AFB_1		ND		<	52%		<	70%	>*	
	AFG_1		—		<*		14%	>		<	53%
9–12	AFB_1	42%	>		ND			<	67%	>	57%
	AFG_1		<	51%	>*			<	21%	>	73%

Comparison of quantities of HCHO produced from AFB_1 and AFG_1 *O*-demethylation between the three experimental groups.

For description of groups I–III, see Table 1.

ND, No difference between the quantity of HCHO produced.

* Used when only one dietary group produced HCHO.

< (I–II) = Higher in group II by the percentage shown.

< (II–III) = Higher in group III by the percentage shown.

> (I–II) = Higher in group I by the percentage shown.

> (II–III) = Higher in group II by the percentage shown.

Table 5. Comparative demethylation activity between male and female within the same dietary group

		I			II			III		
		Male	vs	Female	Male	vs	Female	Male	vs	Female
4 weeks	AFB_1	59%		>	<		54%	90%		>
	AFG_1			<*	<		91%	<		22%
8 weeks	AFB_1	60%		>	<		19%	>*		
	AFG_1			<*	<*			<		17%
12 weeks	AFB_1	31%		>	<		72%	<		36%
	AFG_1	57%		<	<		31%	<*		

Comparison of quantities of HCHO produced from AFB_1 and AFG_1 of the three experimental groups between males and females.

For description of groups I–III, see Table 1.

> Higher in male animals by the percentage shown.

< Higher in female animals by the percentage shown.

*Used when one sex demethylates only.

vitamin C) the quantities of HCHO produced in males are 90 per cent higher than in females. At 8 weeks the pattern is similar to those at 4 weeks, except that the enzyme activity is 19 per cent lower in control males (1.08 mg/ml vitamin C). At 12 weeks, in the first group (no vitamin C offered in the water), the activity in male is 31 per cent higher than in female. That value dropped by half when compared with those at 4 and 8 weeks. But in the second group the *O*-demethylase activity is 72 per cent higher in the female than in the male, and in the third group it is only 36 per cent higher.

Aflatoxin G_1 *O*-demethylation at 4 weeks shows with female rats that activity is 91 per cent higher in group II when compared to group I or group III (Table 4). At 8 weeks, animals of group III demethylate AFG_1 more efficiently, the female rats show a value of HCHO produced of 53 per cent higher than in group II. At 12 weeks, the group II animals have a higher percentage of demethylase

Table 6. Comparative activity of demethylase activity during the period of vitamin C intake (between 4 weeks to 12 weeks)

		I		II		III	
		Male	Female	Male	Female	Male	Female
Between 4 and 8 weeks	B_1	51%	54%	50%	14%	20%	—
	G_1	—	91%	—	ND	16%	18%
Between 8 and 12 weeks	B_1	51%	75%	26%	74%	32%*	V(100%)
	G_1	V(100%)	18%	—	45%	—	53%
Between 4 and 12 weeks	B_1	80%	87%	64%	78%	29%*	92%
	G_1	V(100%)	94%	V(100%)	48%	—	83%

Comparison of quantities of HCHO produced from AFB_1 and AFG_1 *O*-demethylation of the three experimental groups.

For description of groups I–III, see Table 1.

The quantities of HCHO produced from AFB_1 *in vitro* with hepatic microsomes plus 9000 *g* supernatant of rats at 4 weeks are compared with HCHO produced similarly *in vitro* with rats of 8 and 12 weeks respectively.

ND, No difference between the quantity of HCHO produced at 4 and 8 weeks.

V, Increase at 8 weeks or 12 weeks.

* Decrease of demethylase activity.

Table 7. nmoles AFB_1 metabolized/hr/mg liver supernatant protein

Groups		4 Weeks	8 Weeks	12 Weeks
I	Male	0.081* ± 0.025	0.0181* ± 0.025	0.204* ± 0.029
	Female	0.067 ± 0.015	0.167* ± 0.013	0.859* ± 0.032
II	Male	0.117 ± 0.015	0.209 ± 0.061	0.299 ± 0.092
	Female	0.072 ± 0.019	0.11 ± 0.041	0.300 ± 0.015
III	Male	0.217* ± 0.057	0.29* ± 0.022	0.236* ± 0.029
	Female	0.081* ± 0.020	0.061* ± 0.012	0.0115* ± 0.007

Liver of rats were pooled for each dietary sub-group according to sex. Five replicate assays of hydroxylation were made as indicated in the text.

Values represent mean ± S.E.M. of three experiments.

* Shows values which are significantly different from control (group II) at $P < 0.05$.

For description of groups I–III, see Table 1.

Table 8. Comparative summary of Table 7 (AFB_1 hydroxylation)

	Male					Female				
	I	–	II	–	III	I	–	II	–	III
4 weeks		<	25%	<	46%		<	7%*	<	11%
8 weeks		<	13%	<	27%	34%	>	44%	>	
12 weeks		<	31%	>	21%		<	13%	>	96%

For description of groups I–III, see Table 1.
< (I–II) = Higher in group II by the percentage shown.
< (II–III) = Higher in group III by the percentage shown.
> (I–II) = Higher in group I by the percentage shown.
> (II–III) = Higher in group II by the percentage shown.
* Not significant at $P < 0.05$.

activity: 51 and 21 per cent respectively in male and female compared to animals which were fed without vitamin C.

Intersex comparison (Table 5) of AFG_1 demethylation shows that, at all ages studied, activity in the female is greater than in the male. For example at 4 and 8 weeks the activity of female is 22 and 17 per cent higher respectively than that of male in group III.

Table 6 is a comparison of demethylase activity between the times 4–8 weeks, 8–12 weeks and 4–12 weeks for aflatoxin B_1 and G_1 demethylation. Percentages shown in the table within the first, second and third groups represent increases. When the value indicates a decrease within these respective groups, an asterisk has been used. For example within the first group (fed without vitamin C), there is 51 per cent increase in HCHO produced from 4 to 8 weeks. But in the third group (5.4 mg/ml vitamin C) there is a decrease of 32 per cent from 8 to 12 weeks.

Table 7 shows the result of the hydroxylation study *in vitro* of AFB_1. This table has been simplified in Table 8 to show percentage increase or decrease in activity of the hydroxylation. Within 4 weeks, male rats receiving excess vitamin C (group III) have a score of 46 per cent, 21 per cent higher than that of group II, which in turn is 25 per cent higher than in group I. In the female groups at 4 weeks the pattern is similar except for the low values of 11 and 7 per cent obtained. At 8 weeks, the patterns of hydroxylation in male and female are entirely different. While hydroxylation activity increases from the group receiving no vitamin C to excess vitamin C group in the male, it increases from excess vitamin C to the group fed without vitamin C in the female. With 12 weeks, in the male, the activity is 21 per cent lower in excess vitamin C group than group II, which is 31 per cent higher than in the group fed without vitamin C. Female animals show 96 per cent less activity in excess vitamin C group than in group II. This latter is 13 per cent higher than in the group of animals fed without vitamin C.

Table 9 compares hydroxylase activity in males and females inside the same dietary group. In general, male hydroxylation activity is higher than in female.

DISCUSSION

In view of the growing body of theoretical, epidemiological and practical evidence, suggesting that the availability of vitamin C is the determinant factor that regulates various aspects of host resistance to cancer [13], and since AFB_1 is one of the potential carcinogens in foods, we decided to study the effect of vitamin C on aflatoxin metabolism.

The transformation of potential carcinogens to ultimate carcinogens is mediated by the mixed function oxidase, MFO (monooxygenases) whose terminal factor cytochrome P-450 [26] has been implicated in colon carcinogenesis [21]. The hydroxylation activity with AFB_1 as well as *O*-demethylation with male rats, show a positive response with vitamin C, increasing from 4 to 12 weeks. Similar effects of vitamin C in hydroxylation activities of liver microsomes on aniline, hexobarbitone and zoxalamine have been reported by Kato *et al.* [27]. Although the same results can be observed with females up to 4

Table 9. Comparative hydroxylation activity of males and females inside the same dietary group

	I			II			III		
	Male vs Female			Male vs Female			Male vs Female		
4 weeks	24%	>		35%	>		62%	>	
8 weeks		ND		47%	>		78%	>	
12 weeks		<	21%		ND		94%	>	

For description of groups I–III, see Table 1.
ND No difference between the quantity of HCHO produced by the two sexes.
> Higher in male animals by the percentage shown.
< Higher in female animals by the percentage shown.

weeks, the hydroxylation is better with male rats than females. This observation supports the view of Gurtoo [28] who demonstrated that the hydroxylated metabolites of AFB_1 by hepatic microsomes derived from male rats was two and a half times greater than that produced from microsomes of females.

The *O*-demethylase enzyme responsible for the side shain single carbon metabolism of the methoxy group of aflatoxin B_1 is important because this enzyme system can be classified as one of the MFO's. The activity of *O*-demethylase is directly related to the vitamin C content of the diet in experimental male and female Wistar rats. Similar observations have been made in guinea pigs [2, 6, 11]. This similarity is significant because the metabolism of vitamin C, *per se*, in the species does not have the same pathway.

The reaction in which we were interested, namely *O*-demethylation, may be accompanied by other side reactions. For instance, vitamin C can directly oxidise aromatic hydrocarbons *in vitro* [29]. This reaction, if feasible under our *in vitro* system, may affect demethylation by considering mesomeric effect and electron resonance in the aflatoxin ring system. Demethylation and hydroxylation studies which have been reported in guinea pigs [2, 6, 11] are in consonance with our findings and it is now necessary to study other side reactions which ascorbate directs under these *in vitro* conditions.

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