Table 2. Analysis of mepyramine inhibition curves as binding to two independent sites: percentage and IC_{50} of high affinity site*

[^{3}H]Mepyramine concentration (nM)	High affinity site		Expected
	%†	IC_{50} (nM)	IC_{50} (nM)‡
Expt. 1			
1.5	91 ± 2	0.9 ± 0.1	2.1
2.8	88 ± 3	2.8 ± 0.5	3.4
Expt. 2			
1.5	76 ± 9	1.2 ± 0.5	2.1
4.3	85 ± 10	4.2 ± 1.2	4.9
8.4	81 ± 9	7.4 ± 2.5	9.0
Expt. 3			
5.0	64 ± 9	4.9 ± 1.6	5.6

* Values for the IC_{50} and percentage of the binding of [^{3}H]mepyramine associated with the high affinity site were obtained by fitting a double hyperbola to the experimental data using the non-linear minimization routine, VBO1A, as described in the Methods.

† As a percentage of the antagonist-sensitive binding.

‡ Values expected for an interaction with the histamine H_1 receptor with an affinity for mepyramine of $1.8 \times 10^9\ M^{-1}$ [8].

there may be a heterogeneous population of binding sites characterized by differing affinities for mepyramine in guinea-pig cerebral cortex. The increased labelling of secondary low affinity, presumably non-H_1 receptor, sites would contribute to the low Hill coefficients and the reduction in values of K_a observed at high [^{3}H]mepyramine concentrations. For those inhibition curves with Hill coefficients significantly less than unity the data have also been fitted to a two-site model as described in the Methods. The values for the percentage, expressed relative to the antagonist-sensitive portion of the binding, and the IC_{50} associated with the high affinity site are set out in Table 2. It is notable that the IC_{50} values (Table 2) appear to increase linearly with the concentration of [^{3}H]mepyramine (correlation coefficient, 0.99) and are close to the values expected for an interaction with the histamine H_1 receptor.

An alternative explanation to the two site model might be that the high concentrations of mepyramine required to define the foot of the inhibition curve have membrane or other effects which influence the conformation of the receptor. The increased competition with [^{3}H]mepyramine at higher [^{3}H]ligand concentrations would shift the inhibition curve to higher inhibitor concentrations at which such secondary phenomena may become more evident.

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It is apparent that most of the binding at low concentrations of [^{3}H]mepyramine (i.e. 1 nM, the concentration normally employed in this type of study) is to sites with the character of histamine H_1 receptors. There is a good agreement between the affinity constants determined for a range of ligands from the inhibition of the binding of [^{3}H]mepyramine and those obtained from inhibition of histamine H_1-mediated responses [3, 4, 8]. However, at higher concentrations of [^{3}H]mepyramine there appears to be a significant low affinity component of binding resulting either from a separate population of binding sites or from some secondary pharmacological effect which decreases the apparent binding affinity of [^{3}H]mepyramine to homogenates of guinea-pig cerebral cortex. This is evident in the present study where the mean value for the K_a of mepyramine, obtained from three separate experiments at high [^{3}H]ligand concentration (8–10 nM), was $4.7 \pm 2.6 \times 10^7\ M^{-1}$, significantly different from the value of $1.8 \times 10^9\ M^{-1}$ reported for inhibition of histamine H_1-mediated responses in guinea-pig cerebral cortex [8].

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Hydroxylation activity of aflatoxin B_1, and effect of vitamin C on rabbits

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Aflatoxins, which are hepatocarcinogens produced by *Aspergillus flavus*, have been shown to be metabolized by the mammalian hepatic mixed-function oxidase (MFO) system to a number of oxidized products which are less carcinogenic and less toxic than the parent compounds [1]. Several workers have shown that vitamin C deficiency results in a decreased metabolism of many drugs [2, 3]. In order to find out the possible effect of ascorbic acid on the appearance of aflatoxin metabolites, a series of experimental studies have been carried out with laboratory animals susceptible to aflatoxin toxicity and carcinogenesis [4, 5]. We have therefore studied the effect of vitamin C

supplementation on aflatoxin B_1 (AFB_1) hydroxylation in rabbits.

Experimental procedure

Animal treatment. Male and female weaning rabbits (800–1000 g) were utilized. The animals were fed a normal diet for 3 days for acclimation. They were then randomized and divided into three groups with an equal number of males and females in each dietary group. Separate cages were used for each animal. All groups were fed an experimental diet previously described with a salt mixture by Sure [6], and with a vitamin mixture except vitamin C by Chapman *et al.* [7], as modified by Becking [8]. While group 1 was offered no vitamin C in the drinking water, group 2 received 1.08 mg vitamin C/ml and group 3, 5.4 mg vitamin C/ml. Vitamin C was prepared freshly every day.

Ascorbic acid ingested per day in group 2 was 237.6 ± 10.8 mg for males and 199.8 ± 16.2 mg for females; in group 3 it was 594.4 ± 32.7 mg for males and 765.0 ± 60.2 mg for females. Animals were maintained on the basal diet and vitamin C doses for 30 days. Twenty-four hours after the last feeding the animals were killed by decapitation. The livers of animals in each dietary group, according to sex, were homogenized at 0–4° in 0.3 M phosphate buffer (pH 7.4) using a Waring Blender. The hepatic 9000 *g* supernatant was prepared by a method previously described [9].

Enzyme assay. Liver supernatant protein was determined by the biuret method [10], and the quantity of protein expressed as mg/ml of supernatant. Bovine serum albumin (Knoch-Light) was used as the standard. Enzyme assays were carried out with Sigma reagents. The method previously described [11] was slightly modified. 9000 *g* supernatant aliquots equivalent to 0.5 g of fresh liver were incubated with AFB_1 (50 nmoles in DMSO), in a medium consisting of potassium phosphate buffer (0.1 M, pH 7.4), $NADP^+$ (3 μmoles), glucose 6-phosphate (50 μmoles), nicotinamide (50 μmoles) and magnesium chloride (25 μmoles). The total volume per flask was 5 ml. The incubation time was 1 hr at 37° in a Gallenkamp shaking incubator. The hydroxylase assay was terminated by addition of 5 ml of saturated NaCl followed by 5 ml of cold chloroform. The mixture was stored for no longer than 12 hr at 4–6° before being extracted 5 times with 10 ml of chloroform. The total extract was then concentrated to approximately 0.5 ml in the evaporator at 40° prior to spotting on activated thin-layer chromatographic plates covered with silica gel G (Hopkin & Williams). The crude extracts were spotted alongside AFB_1 and AFM_1 standards (Makor Chemicals, Jerusalem). The latter, after developing in ethyl acetate–chloroform (2:1), were identified under u.v. light. The AFB_1 bands, (R_f 0.5) and aflatoxin M_1 (R_f 0.3) were characterised as well as aflatoxicol (R_f 0.66), otherwise called AFR_0, which appeared to be the main *in vitro* metabolite of AFB_1 in rabbit liver [12]. The individual metabolites were eluted from their respective silica gel scrappings by washing with methanol. The quantities of AFB_1, AFM_1 and AFR_0 so eluted were calculated from their absorbance at 363, 357 and 325 nm respectively [13]. The specific activity was expressed as nmoles of metabolites produced/hr/mg supernatant protein.

Vitamin C content in the liver was estimated by the method of Roe and Kuether [14].

Results and discussion

The effect of vitamin C intake on the liver vitamin C content is shown in Table 1. Rabbits in group 2 have the greatest ascorbic acid content in the liver: 110 and 31% higher than group 1 and 3 females respectively; 95 and 40% higher than group 1 and 3 males respectively. Many workers using guinea pigs [15, 16] have suggested that a high intake of vitamin C increased the amount of vitamin

Table 1. Effect of vitamin C intake on liver vitamin C content, body weight and AFB_1 metabolites by *in vitro* hydroxylation assay

Dietary vitamin C (mg/ml)	Group 1 0	Group 2 1.08	Group 3 5.4
Vitamin C in the liver (μg/g)			
Females	14.89 ± 0.82	31.19 ± 1.41	23.99 ± 1.82
Males	23.44 ± 1.32	43.77 ± 2.25	31.12 ± 2.80
Vitamin C in the liver			
Vitamin C intake (μg/100 mg)			
Females		48.64 ± 2.82	5.88 ± 1.14
Males		43.16 ± 1.32	3.24 ± 0.64
Liver weight (g)			
Body weight (100 g)			
Females	2.10 ± 0.10	2.19 ± 0.13	2.24 ± 0.20
Males	1.90 ± 0.08	2.40 ± 0.10	3.08 ± 0.12
AFB_1 metabolites:AFR_0 (nmoles/hr/mg protein)			
Females	1.003 ± 0.700	0.756 ± 0.085*	0.293 ± 0.050*
Males	0.619 ± 0.071	0.427 ± 0.082*	0.217 ± 0.040*
AFB_1 metabolites:AFM_1 (nmoles/hr/mg/protein)			
Females	0.531 ± 0.069	0.422 ± 0.025	0.305 ± 0.005
Males	0.306 ± 0.005	0.276 ± 0.007	0.192 ± 0.020

Assay methods are described in Experimental procedure. Data represent means ± S.E. for three experiments with four to seven animals per group and sex.

* Statistical significance between dietary treatments is indicated by * for P = 0.05 (Student's *t*-test).

C in the tissues, and accelerated its metabolism or excretion. As with the findings of the earlier workers [15, 16], the relation of vitamin C in the liver vs vitamin C intake (Table 1) confirmed that animals in group 2 fed 1.08 mg vitamin C/ml retained more vitamin C in their livers than did those, in group 3, fed a high level (5.4 mg vitamin C/ml).

Table 1 shows also the hydroxylation activity expressed as the amount of metabolites obtained. The quantity of AFR_0 in group 1 females deficient in dietary vitamin C was 32% higher than for those in group 2, and the latter was 158% greater than for those in group 3 on a high-level dietary vitamin C. With males the increased doses of dietary vitamin C also decreased the amount of AFR_0 formed by the hepatic 9000 *g* supernatant. Group 1 had 45% more AFR_0 than group 2, and the latter had 97% more than group 3. The AFM_1 formed was less affected by vitamin C intake than AFR_0. However, the amount of metabolites (AFR_0 or AFM_1) obtained from females was higher than from males in each dietary group: in group 1, for example, AFR_0 produced in males was 38% lower than in females.

In conclusion a high vitamin C level intake (5.4 mg/ml) mainly decreased the production of AFR_0; that effect was more obvious in female than in male rabbits.

From previous work [17] it has been shown in male rabbits that AFB_1 hydroxylase activity tended to be higher in the vitamin A deficient animals compared to controls. A similar effect was observed with vitamin A deficiency on benzopyrene metabolism [18, 19]. Our studies on the vitamin C effect on the hydroxylase activity of AFB_1 in male and female rabbits showed an increased activity in the group fed without vitamin C (group 1), and a decrease in the group fed with high-level ascorbic acid (group 3). These results correlated with previous findings [17–19]. The similarity of the effects of these two vitamins (A and C) can be explained by the fact that the vitamin C status of an animal is considerably influenced by its vitamin A status. Vitamin A deficiency lowered the ascorbic acid content of several tissues including liver and blood plasma [20, 21]. Elsewhere, in spite of the fact that male rabbits retained more vitamin C in their liver than did females (Table 1), their ability to produce AFR_0 or AFM_1 was less than in females. This second observation as well as the first seems to be completely different from the results obtained with rats [4, 22], animals which, like rabbits, also synthesized their required vitamin C. Thus, more work has to be done to clarify these two observations. There has therefore been reported a marked variation in the response to aflatoxins between various species of animals [23]; and a qualitative change in the metabolism of the toxin rather than quantitative differences in a single enzyme may be an explanation [24]. Consequently different species of animals may deal with a foreign compound in different ways. This is the case with, for example, the dog which hydroxylates amphetamine, while the rabbit deaminates it [24]. It appears that the metabolism of AFB_1 into AFR_0 essentially, which is catalyzed by NADPH-dependent 17-hydroxysteroid dehydrogenase [25], is reduced by a high intake of vitamin C. Many workers using guinea pigs have demonstrated an effect of vitamin C on cytochrome P-450, which is the terminal factor of the MFO system, responsible for the metabolism of drugs and aflatoxins [26, 27]. Further studies have to be carried out to clarify also the effect of vitamin C on the enzyme responsible for the production of AFR_0 from AFB_1.

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