18-20 °C, since pronounced temperature variations were found to alter the duration of THAO tremor. Mice were housed in groups of 12 and randomly assigned to the treatment groups. 6 mice were used in each group. The presence of tremor was assessed by observation of the 4 limbs when the mice were lying on their side and when held up in the cupped hand. The degree of tremor for each animal was observed by a trained observer who was unaware of the pretreatment regimen and scored on a scale of 1-3, with 1 representing mild tremor and 3 the most severe tremor. The mice were also observed for loss of righting reflex, ptosis and other vital signs. The statistical significance of the differences in duration of tremor was assessed by the Student's nonpaired t-test. Thiophene-2-carboxaldehyde oxime was purchased from Aldrich Chemical Company, Inc. It was dissolved extemporaneously in dimethylsulfoxide to make a 10% solution and injected i.p. in volumes not exceeding 0.1 ml. Control animals given an equivalent volume of solvent showed no tremor. All other drugs were prepared as fresh solutions in saline and injected i.p., except haloperidol (Haldol), reserpine (Serpasil), Clonidine (Catapress) and chlorimipramine (Anafranil) where proprietary parenteral solutions were used.

The results are summarized in the table. A dose of THAO (175 mg/kg) which was found in previous experiments[2] to be the threshold dose which produced tremor and loss of righting reflex in all mice (ED 100) was administered to saline-pretreated mice. These served as controls. Pretreatment of mice with agents which are known to alter the content of serotonin or its actions in the central nervous system (5-hydroxytryptophan, p-chlorophenylalanine, cinanserin) failed to affect the intensity and duration of tremor significantly.

A wide spectrum of substances which interact with brain catecholamines was tested. Agents which deplete catecholamines (alpha-methyl p-tyrosine, reserpine) or antagonize their central actions (haloperidol, chlorpromazine) significantly increased the intensity and duration of the tremor and the loss of righting reflex. Conversely, pretreatment with agents which enhance catecholaminergic tone (apomorphine, pargyline, d-amphetamine, chlorimipramine, ephedrine, L-dihydroxyphenylalanine, but not clonidine) reduced the duration and intensity of tremor. The most effective agent, aside from d-amphetamine, was apomorphine. In view of the evidence for dopamine receptor stimulation by apomorphine[5], its antagonism of THAO tremor implicates a dopaminergic mechanism. This is further supported by the enhancement of this tremor by haloperidol, an agent shown to possess marked dopamine receptor blocking action[6]. However, an adrenergic component may also be operative, since the monoamine oxidase inhibitor pargyline and the antidepressant chlorimipramine were moderately effective in reducing the tremor.

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Kinetics of transformation of aflatoxin B_1 into aflatoxin M_1 in lactating mouse: An ELISA analysis

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Summary. A new enzyme-linked immunosorbent assay (ELISA) was used to study the kinetics of transformation of aflatoxin B_1 into aflatoxin M_1 in lactating mice. Aflatoxin M_1 concentration in the milk samples reached a maximum 30 min after injection of aflatoxin B_1 and decreased thereafter. At the maximum time, the levels of aflatoxin M_1 in the samples were proportional to the dosages administered. Aflatoxin B_1 was also detected in the milk samples but at a lower concentration.

Aflatoxin M_1 (afla M_1) is one of the major aflatoxin B_1 (afla B_1) metabolites produced by a number of animal species and has been found in animal livers, urine, feces and milk[3-7]. Since afla M_1 was found to be toxic as well as carcinogenic to test animals[8-10], the presence of this toxin in cow's milk has been considered to be potentially hazardous to human health. A rigorous regulatory program for afla M_1 in foods has been established in the U.S. as well as in other countries. The extent of afla M_1 contamination in dairy products and the impact of afla M_1 on human and animal health have been recently reviewed[11].

Since the discovery of excretion of afla M_1 in the milk of cows consuming afla B_1 contamined feeds, considerable efforts have been made by various investigators to elucidate the mechanism and kinetics of transformation of afla M_1 in to afla B_1 in animals and its subsequent excretion into the milk[12,13]. Most excretion studies were done with large animals including cows, sheep and ewes[7,11-13]. Studies with small animals such as rats[14] and mice were hindered by the unavailability of a suitable analytical method to detect these metabolites in a small amount of milk. Recent developments on the immunochemical assay of aflatoxins in our laboratory and others[15-18] have led to a sensitive, simple and specific microplate enzyme-linked immunosorbent assay (ELISA) for afla M_1 in which as little as 0.25 ppb of afla M_1 in a 25 µl of milk sample can be readily detected[18]. With the availability of this method, we have selected mouse as the test animal to carry out the present study. The objectives of this study are 2-fold: a) to test the feasibility of using this new ELISA procedure for metabolic studies and b) to analyze the kinetics of transformation of afla B_1 into afla M_1 in the lactating mice.

A colony of lactating mice (strain-HA/ICR, Sprague Dawley, Madison, WI) with an average size of 50 g was divided into 2 groups (4-5 mice each). Mice in the 1st group were injected i.p. with 5 µg each of pure afla B_1 prepared according to the method of Chu[19] and mixed with a small amount of ^{3}H-afla B_1 (Moravek Biochemicals, City of Industries, CA) in 0.1 ml dimethylsulfoxide. The

specific activity of the final mixture was 1400 dpm/ng afla B_1. In the 2nd group, a dose of 50 µg afla B_1 without ^{3}H-afla B_1 was used for each. Milk samples were collected before administering afla B_1 and at appropriate times after injection using the procedure of Nagasawa[20] except that no anesthesia was applied to the test animals before milking. For each mouse, approximately 0.2 ml of milk was collected every time. The milk samples from different mice were pooled, and if necessary diluted to an appropriate concentration (0.5–1.0 ng/ml) with sodium phosphate buffer (0.1 M, pH 7.4) and then subjected directly to the ELISA according to the procedures previously described[18]. For afla B_1, antisera against afla B_1 and horseradish peroxidase conjugated with afla B_1 were used[16]. Likewise, afla M_1 antisera and horseradish peroxidase conjugated with afla M_1 were used in the ELISA of afla M_1 (Pestka et al.[18]). Triplicate analyses were made for each sample. The radioactivity of the samples was measured in a Beckman Model LS 330 scintillation counter and corrected for background and counting efficiency (25–35%) by a channels ratio method.

Results for the transformation of afla B_1 into afla M_1 in the lactating mouse after injection of afla B_1 are given in the table. To test the validity of our ELISA data, mice in 1 group were injected with ^{3}H-afla B_1. Aflatoxins were extracted from the 30 min milk sample with methanol: chloroform (1:9 ratio, 10× vol., 2 times), and then subjected to TLC using adsorbsil 5 as the absorbent (Applied Science, State College, PA, USA) and developed in chloroform: acetone (9:1). External standards of afla B_1 and M_1 were spotted simultaneously with the sample. After development, materials in the afla B_1 and M_1 spots were scraped from the plates and the radioactivity determined. The results showed that 4.2 ng/ml (5862 dpm/ml) of afla M_1 and 2.2 ng/ml (3125 dpm/ml) afla B_1 were present in the 30 min sample. The concentration of afla M_1 determined by the TLC method was approximately 60% of that of the ELISA. The lower value obtained by the TLC method might be due to losses in the extraction step and/or to using an inadequate specific radioactivity in the estimation. Since excellent recovery of afla M_1 added to cow's milk has been obtained by ELISA in our previous investigations, we believe that the ELISA result is more accurate than the TLC method.

In the present study, we found that afla M_1 concentration in the milk reached the maximum 30 min after injection of 50 µg of afla B_1 and decreased thereafter. The amount of afla M_1 in the 30 min milk samples appears to be directly proportional to the dosage administered. The maximum concentration of afla M_1 (63.3 ng/ml) in the milk of mice injected with 50 µg of afla B_1 per mouse (1000 µg/kg b.wt) is almost 10 times higher (63.3 vs 7 ng/ml) than those injected with 5 µg. Based on these data, the relationship between the maximum afla M_1 concentration in milk and the amount of afla B_1 administered was estimated to be 60–70 ng afla M_1 per ml with every mg afla B_1 per kg b.wt administered. It is interesting to note that this value is similar to the data obtained from cows. Allcroft et al.[12] found that the highest amount of afla M_1 in cow's milk was around 14–15 ng/ml when a cow of 600 kg size was fed a single dose of aflatoxin mixture containing 132 mg of afla B_1 (220 µg afla B_1/kg b.wt). Although the present data cannot be directly compared to the study with cows because of the difference in route of administration and the species difference, as the cow has a rumen and the degradation of afla B_1 occurs in the rumen and omasum[21], the similarity of the maximum afla M_1 concentrations in milk further supports the hypothesis that 'liver' is the major organ for conversion of afla B_1 into M_1 and that the cow rumen does not play a significant role in this transformation.

In conclusion, the present study demonstrates that the ELISA for afla B_1 and M_1 developed in our laboratory is adequate for the metabolic study of conversion of afla B_1 to M_1. Since only a small amount of sample is required for the analysis, this approach could be used to monitor afla M_1 and B_1 in human milk in certain areas where afla B_1 has been found in food and where a high incidence of liver cancer has been documented. Direct epidemiological correlation between liver cancer incidence and aflatoxin consumption might then be obtainable. Since significant amounts of afla B_1 have been detected in mice, the lactating mouse and weanling mouse could be used as an animal model system to study the effect of afla B_1 and M_1 on weanling animals.

Recovery of aflatoxins B_1 and M_1 in mouse milk after administration of aflatoxin B_1 to lactating mice

Time (h)	Dose A (5 µg/mouse) Afla M_1 (ng/ml)	Dose B (50 µg/mouse) Afla M_1 (ng/ml)	Afla B_1 (ng/ml)
0	0	0	0
0.5	7.0±0.6	63.3±4.3	--[a]
1	--	54.0±1.1	7.0±0 (6.4)[b]
3	2.0±0.2	32.0±1.7	5.4±0.5 (5.9)
5	--	12.8±1.7	--
8	0.7±0.0	--	--
10	--	12.0±2.3	3.1±0.7 (4.0)
24	0.6±0.1	1.6±0.1	--

[a]Not analyzed; [b]values in parentheses in this column indicate the ratio of afla M_1/afla B_1.

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