Dietary Lipotropes, Hepatic Microsomal Mixed-Function Oxidase Activities, and in Vivo Covalent Binding of Aflatoxin B₁ in Rats

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ABSTRACT

Weanling male Sprague-Dawley rats were fed either a nutritionally complete synthetic diet (Diet 1) or a diet marginally deficient in choline and methionine, and lacking folacin (lipotrope deficient, Diet 2) to determine the role of hepatic mixed-function oxidase metabolism of aflatoxin B₁ (AFB₁) in the Diet 2-induced enhancement of AFB₁, hepatocarcinogenesis previously reported. Hepatic microsomal mixed-function oxidase activities, as assayed by ethylmorphine N-demethylation, ethoxycoumarin O-dealkylation, cytochrome c reduction, AFB₁ metabolism, and cytochrome P-450 content, were all depressed by Diet 2.

Furthermore, the proportion of an i.p. dose of AFB₁ (1 mg/kg) that became covalently bonded to DNA and RNA was similarly reduced when measured 6 hr after administration. The formation of AFB₁-protein adducts was not influenced by dietary treatment. The depression of DNA and RNA adduct formation in the Diet 2 animals was probably related to the lower mixed-function oxidase activities and not to an alteration of glutathione levels, which remained unchanged by dietary treatment. These results suggest that the marginally lipotrope-deficient diet does not enhance tumor formation through an increased microsomal activation of AFB₁. Alternative hypotheses without data are suggested.

INTRODUCTION

Nutrition and/or dietary practices have been suggested to be responsible for 40 to 60% of human cancers in western societies when epidemiological data are considered. This, together with the previous hypothesis that 80 to 90% of human cancer is related to chemicals and environmental factors (2, 8, 18), suggests that the quantitative and qualitative character of nutrient intake may play important roles in establishing the potential of chemical carcinogens. Since most chemical carcinogens of current concern require metabolic activation by the MFO enzyme system and this activity may be readily modified by nutrient level and composition (5), it becomes of interest to study the effect of nutrient intake on the metabolic activation of chemical carcinogens. Of the multifactorial events that may be required for full tumor development, the initial rate of MFO activation to yield macromolecular adducts may be one of the more important. There are several reports that have demonstrated a relationship between nutrient intake and chemical carcinogen activation (29, 30, 34), but one of the original ones was that concerning the effect of marginally deficient lipotrope diets on AFB₁, hepatocarcinogenesis and metabolism (31).

Rats are more susceptible to AFB₁-induced hepatocarcinogenesis when fed high-fat diets marginally deficient in the lipotropes methionine, folacin, and choline (31). Such a deficiency has been shown to depress in vitro hepatic microsomal MFO activity required for AFB₁, metabolism (29, 32). That MFO activity is similarly depressed in vivo is suggested by the finding that clearance of diethylnitrosamine from the blood and liver of rats is depressed (33) and barbiturate sleeping time is prolonged by lipotrope-deficient diets (21). It has therefore been suggested that the depression of MFO activity may alter AFB₁, metabolism in lipotrope-deficient animals (27, 32) and presumably may increase the proportion of an AFB₁ dose that becomes activated to the ultimate carcinogen.

The activated AFB₁ metabolite covalently binds the nucleic acid nucleophiles in rat liver, and the magnitude of this binding has been shown to be related to tumorigenic potential. For example both barbiturate treatment (24) and protein-deficient diets (23, 36) depress tumor yield; likewise, both treatments reduce the quantity of DNA- and RNA-AFB₁ adducts formed in vivo (9, 30). This observation is in spite of the rather anomalous behavior of the MFO system, which is induced by barbiturate treatment but depressed by inadequate protein intake (4).

This study was therefore undertaken to determine whether the reported effect of lipotrope deficiency on AFB₁, tumorigenesis could be related to its MFO-mediated metabolism to a product that covalently binds macromolecules.

MATERIALS AND METHODS

Animals and Diets. Male, weanling Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used. Animals were placed on their respective diets for 90 days after receipt at the Massachusetts Institute of Technology, then shipped to Cornell University, and fed for another 7 to 10 days prior to sacrifice. There were 2 separate shipments that were spaced about 1 year apart.

The data presented in this report were in agreement for each shipment and were therefore grouped for this report. The composition of the diets that were adequate (Diet 1) and those that were marginally deficient (Diet 2) in lipotropes are presented in Table 1.
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**Tissue Preparation.** Upon sacrifice livers were removed, weighed, and perfused with cold 0.9% NaCl solution to remove blood. The entire liver was then homogenized in 2 volumes of 0.2 M Tris buffer containing 1.15% KCl. A mechanically driven Potter-Elvehjem homogenizer was used for the homogenization, with the use of 600 rpm and 6 complete strokes. Aliquots of the homogenate were removed for the assays noted later; the remaining portion was used to isolate microsomes as described previously (15). All tissue preparations were carried out at 0-5°C.

**Determination of Liver Constituents.** Aliquots of the liver homogenate were used for assay of RNA by the method of Ceriotti (6), DNA by the procedure of Hubbard et al. (19), GSH by the method of Boyland and Chasseaud (3), total protein either by the procedure of Lowry et al. (22) or by the method of Gornall et al. (12), and total lipids by the method of Hayes et al. (16).

**MFO Assays.** The following measurements on MFO enzyme activities were carried out on microsomal suspensions of the liver homogenate. The microsomal suspension was produced by the method previously used in our laboratory (15). MFO activities were estimated with saturating substrate concentrations after linearity was assured with incubation time. For measurement of these activities ethylmorphine N-demethylation was determined by the method of Davies et al. (7), ethoxycoumarin O-dealkylation by the method of Ullrich and Weber (35), cytochrome c reduction by the procedure of Gigon et al. (10), cytochrome P-450 content by the method of Omura and Sato (28), and AFB, metabolism by the procedures reported by Hayes et al. (17).

Type 1 substrate binding was determined as a difference spectrum with ethylmorphine according to the procedure described by Guarino et al. (13). A substrate concentration range of 0.02 to 50 mM was used in order to estimate both the maximal binding constant (K_m) and the spectral dissociation constant (K_s). The type 1 difference spectrum is characterized by a trough at 419 to 425 nm and a peak at 390 to 405 nm; the substrate-induced difference between the peak and trough was used in this assay.

**Formation of Adducts.** Adduct formation between AFB1, DNA, RNA, and protein was determined by administering a radiolabeled AFB1, dose and then determining the quantity of label covalently binding these macromolecular fractions. [3H]AFB1 (Moravek Biochemicals, City of Industry, Calif.) was diluted with cold AFB1, (Makor Chemical, Jerusalem, Israel) to a specific activity of 7 μCi/μmol and injected i.p. at a dose of 1 mg/kg in N,N-dimethylformamide. Animals were killed 6 hr later, and liver homogenates were prepared as described previously. DNA, RNA, and protein were isolated from the homogenate by the method of Glazer and Weber (11), except that only the first DNA extract was used to determine the covalently bound AFB1, metabolite(s). Selected samples were also extracted for DNA by the method of Kirby et al. (20) for comparison with the method of Glazer and Weber. Although each method gave comparative data, only data obtained by the method of Glazer and Weber are presented in this paper. The isolated fractions were counted in a Beckman LS313 liquid scintillation spectrometer with a Triton-toluene cocktail.

**Statistical Treatment.** Statistical comparisons were made by the nonparametric analysis of Wilcoxon and Wilcox (37).

### RESULTS

The marginal deficiency of dietary lipotropes caused a slight increase in liver weight (Table 2). The livers were substantially higher in total lipid but slightly lower in protein. Neither RNA, nor DNA, nor GSH was altered by dietary treatment.

The characteristics of the microsomal MFO enzyme system are shown in Table 3. Cytochrome P-450 content was reduced by 37% in the Diet 2 animals. Generally, we have observed P-450 contents in this rat strain to vary within the limits of 0.4 to 1.1 nmol/mg protein (14, 25, 30) when animals are fed semipurified nutritionally adequate diets.

Microsomal lipid levels were not different and do not reflect the total liver lipid content.

The microsomal catalytic activity was determined by the N-demethylation of ethylmorphine, the O-dealkylation of ethoxycoumarin, and the reduction of exogenously added cytochrome c. All 3 activities were depressed by Diet 2 to a similar extent when expressed on a microsomal protein basis (21 to 39%). When these activities were calculated on the more appropriate body weight basis, the decreases associated with the Diet 2 animals remained approximately the same.

AFB1, metabolism was measured primarily to ascertain potential differences in the patterns of production between 2 of the more significant detoxified products, aflatoxin Q, and aflatoxin M1. None were apparent. The difference in the diets for total AFB1, metabolism suggests a slightly lower rate for Diet 2; however, the total incubation period extended beyond linearity and a more sensitive detection of a significant difference was therefore not possible. Although the variance precluded statistical significance, there were greater quantities of aflatoxin Q, and aflatoxin M1 (both less toxic) in the Diet 2 animals.
Marginal lipotrope deficiency, body growth, and liver composition

Sixteen and 15 animals were fed Diets 1 and 2, respectively, and were assayed in duplicate for liver components.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Final body wt (g)</th>
<th>Wt (g/100 g body wt)</th>
<th>Lipid (mg/g)</th>
<th>Protein (mg/g)</th>
<th>RNA (mg/g)</th>
<th>DNA (mg/g)</th>
<th>GSH (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet 1</td>
<td>326 ± 15</td>
<td>3.3 ± 0.1</td>
<td>41 ± 3</td>
<td>181 ± 4</td>
<td>7.6 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>Diet 2</td>
<td>336 ± 23</td>
<td>4.1 ± 0.1</td>
<td>100 ± 12</td>
<td>157 ± 2</td>
<td>7.2 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>1.3 ± 0.0</td>
</tr>
</tbody>
</table>

*Mean ± S.E.

Marginal lipotrope deficiency and microsomal characteristics

Four to 6 animals were fed Diets 1 and 2, respectively, and were assayed in duplicate.

<table>
<thead>
<tr>
<th>Microsomal protein (mg/g)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
<th>Ethylmorphine N-demethylase (nmol/mg protein)</th>
<th>Ethoxycoumarin O-dealkylase (nmol/mg protein)</th>
<th>Cytochrome c reductase (nmol/mg protein)</th>
<th>AFB, metabolism (ng/mg protein)</th>
<th>MFO type 1 spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet 1</td>
<td>24.4 ± 1.5</td>
<td>1.8 ± 0.1</td>
<td>13.0 ± 1.0</td>
<td>0.350 ± 0.09</td>
<td>66.2 ± 3.7</td>
<td>13.0 ± 0.1</td>
</tr>
<tr>
<td>Diet 2</td>
<td>18.7 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>11.9 ± 1.0</td>
<td>0.215 ± 0.03</td>
<td>52.4 ± 3.5</td>
<td>7.6 ± 0.3</td>
</tr>
</tbody>
</table>

*Statistically significant at p < 0.05.

The type 1 spectral binding data (Table 3) illustrate affinities for type 1 compounds such as those used in the catalysis studies above. Total binding sites are reduced by Diet 2 to an extent (26%) similar to the depressed activities for the MFO enzyme reactions. There was no diet-related difference in the spectral binding constants.

The effect of Diet 2 on the amount of AFB, that became covalently bonded to the tissue macromolecules to form adducts is presented in Table 4. The quantities of DNA and RNA adducts were reduced by Diet 2 to a similar extent, but the formation of protein adducts was not affected by diet. The in vivo affinity of AFB, metabolites for the nucleic acids was considerably greater than for the protein and agrees with previous observations reported elsewhere (9, 30).

### DISCUSSION

The ultimate objective of this study was to ascertain whether a marginal lipotrope deficiency induced greater hepatocarcinogenesis because of an increase in AFB, activation by the MFO enzyme system. The data presented here would suggest that there is no relationship.

The MFO activities were all depressed by Diet 2 as measured by cytochrome P-450 content, 4 enzyme activities (ethylmorphine demethylation, ethoxycoumarin dealkylation, AFB, degradation, and cytochrome c reduction), and the type 1 spectral binding constant. The extent of enzyme depression found here agrees with that found in previous studies (29, 31). Moreover, whole-animal response as measured by diethylnitrosamine clearances (33) and barbiturate sleeping times (21) similarly reflected the depressed MFO activities in the deficient animal. These effects on MFO enzyme activities were similar whether expressed on a microsomal protein basis or on the whole-animal body weight basis.
There was a hint from a previous report (29) that Diet 2 repressed the ability of AFB, to induce MFO activity, although Kula (21) demonstrated that the dietary treatment did not alter induction by phenobarbital. In this study all animals were given injections of AFB, 6 hr prior to sacrifice, and the diet-related differences in MFO activities were still similar to those reported elsewhere (29, 32), with or without AFB, administration.

In addition to the depressed MFO activities, the in vivo formation of covalent AFB,-nucleic acid adducts was similarly depressed. The formation of AFB,-protein adducts was not affected and agrees with previous work that demonstrated the insensitivity of protein adduct formation as an index of AFB, activation (9, 30). The data presented in this paper were obtained from 2 shipments of animals sent to Cornell University from Massachusetts Institute of Technology about 1 year apart. Each shipment presented similar data. The depression of AFB,-nucleic acid adduct formation may be partially explained by the lower quantity of AFB, available in the liver (Table 4), although most of this depression would appear to be due to the lower rate of MFO activation of AFB,. The possibility that altered GSH levels may have modified adduct formation through removal of the activated carcinogen as a mercapturic acid (1, 26) was excluded since GSH levels remained the same in each diet group (Table 2). A similar observation that GSH was not affected by the lipotrope deficiency was reported by Poirier et al. (29). A lower rate of activation by Diet 2 microsomes is also supported by recent work of Suit et al. (34), who showed that activation of AFB, to a mutagenic species was depressed when microsomes from lipotrope-deficient animals were assayed in vitro.

The previous finding (29, 31) that showed the marginally lipotrope-deficient diet depressed MFO activity is thus borne out by these data. Moreover, the lower in vivo MFO activity is associated with a lower MFO activation reaction (34) and with lower levels of DNA adduct formation in these data. This consistency of findings clearly infers that the rate of activation and the quantity of DNA adducts, when measured as total adducts, do not predict eventual tumorigenesis. These results, however, do not rigorously exclude a role for MFO-mediated activation since the measurement of covalent bonding at a single time point after carcinogen administration may not accurately reflect the longer term consequences for neoplastic development. If it is assumed that metabolism of AFB, is not involved in this phenomenon, other hypotheses that might be considered are the possibilities that (a) repair of the adduct is altered and (b) conditions for growth and development of the newly formed neoplastic tissue are modified. Perhaps the depression of the active methyl donor S-adenosylmethionine, which is depressed by Diet 2 (4), plays a role not associated with metabolic activation of AFB,.

Of more general interest is the fact that in vivo measurement of MFO enzyme activity does not always correlate with subsequent tumorigenic potential. This is in spite of the requirement for MFO activation. Clearly, the use of traditional substrates for measurement of in vivo MFO activity may not reflect in vivo MFO activation rates. A more extensive discussion of this obviously tenuous association is presented elsewhere (4). Moreover, measurement of total

in vivo hepatic DNA adducts after 1 dose of the carcinogen does not always reflect tumorigenic potential, as illustrated with these data. A clearer understanding of the kinetic character of adduct formation as it relates to various dosage protocols is required. Also a specific DNA base adduct is undoubtedly more reflective of the principal activation event responsible for tumor development than is total DNA covalent binding. More extensive studies on these latter 2 points are currently under way in this laboratory.

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REFERENCES

Lipotropes and Aflatoxin Metabolism

Dietary Lipotropes, Hepatic Microsomal Mixed-Function Oxidase Activities, and *in Vivo* Covalent Binding of Aflatoxin \( B_1 \) in Rats

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