Cytosol is required for the modulation by dietary casein of the hepatic microsomal activation of aflatoxin B1 to mutagenic metabolites detectable in Salmonella

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We have shown previously that dietary protein (casein) levels can affect the ability of rat liver S9 to metabolize aflatoxin B1 (AFB) as well as other promutagens detectable in Salmonella strain TA98 [Mutat. Res. (1997), 360, 115–126 and 127–143]. The mutagenic potency of AFB was greatest when metabolized by the Aroclor 1254-induced hepatic S9 prepared from F344 male rats that consumed an isocaloric, semisynthetic diet for 6 weeks that contained an adequate (12%) level of methionine-supplemented casein as the sole protein source, compared with S9s from rats fed diets that contained nominally deficient (8%) or high (22%) levels of casein. Here we have extended this observation by performing (i) mutagenicity studies with microsomes, cytosols and reconstituted S9s (recombinations of microsomes and cytosols across dietary groups), and (ii) in vitro incubations followed by analysis of metabolites by fluorescence high-pressure liquid chromatography. Microsomes, but not cytosols, activated AFB; however, activation to the level observed with S9 occurred only when microsomes from the rats fed 12% casein were combined with cytosols from any dietary group. Consistent with the mutagenicity results, the greatest metabolism of the AFB parent compound and the highest level of the glutathione conjugate of the presumptively identified AFB-exo-8,9-epoxide (the ultimate mutagenic form of AFB) were produced by S9s from the rats fed the 12% casein diet. The levels of these metabolites and the mutagenicity of AFB changed in parallel with changes in dietary casein levels. In summary, cytosolic elements, which are not affected by dietary casein levels, interact with microsomal enzymes, which are modulated by dietary casein levels, to influence the ability of hepatic S9 to activate AFB to a mutagen.

Introduction

Nutritional factors can alter the activities of both phase I and phase II enzymes involved in the metabolism of endogenous substrates and xenobiotic compounds (Campbell and Hayes, 1974; Shakman, 1974; Omaye, 1986; Meydani, 1987; Guengerich, 1995). Among these, increasing the level of dietary protein has been shown to exert effects on hepatic esterase activity, hexobarbital sleeping time and bromobenzene hepatotoxicity (Butler and Dauterman, 1988; Eaton et al., 1994). High dietary protein levels induce phase I enzymes in humans; human volunteers maintained on a high-protein, low-carbohydrate diet and then changed to a low-protein, high-carbohydrate diet showed an increase in the biological half-lives of antipyrine and theophylline (Anderson et al., 1982).

In laboratory animals, dietary protein levels can alter toxicant metabolism, the frequency of spontaneous or induced tumors, and life-span (Boyd and Krupa, 1970; Kroes et al., 1986; Butler and Dauterman, 1988). For example, a high level of dietary protein increases the LD50 for a number of pesticides and, conversely, increases the toxicity of many hepatotoxins such as carbon tetrachloride and dimethylnitrosamine (Shakman, 1974). Rats fed 22% casein as a protein source had a higher concentration of P450s and higher P450 activity levels than did animals fed 6% casein (Amelizad et al., 1985). Casein was chosen as the protein in these studies because it is common to many semisynthetic diet formulations and because it was used in previous studies in our laboratory (Butler and Dauterman, 1988).

Aflatoxin B1 (AFB) is an important environmental toxin whose toxic effects may be altered by dietary levels of protein (Eaton et al., 1994). In rodents, increasing levels of dietary protein result in increasing numbers of putative preneoplastic gamma-glutamyltransferase-positive (GGT+) hepatic foci following AFB exposure (Dunaif and Campbell, 1987; Schulsinger et al., 1989; Youngman and Campbell, 1991). Substitution of a poor (lysine-deficient) protein source can reduce the formation of GGT+ hepatic foci following AFB exposure, much the same as reducing the protein content of the diet (Schulsinger et al., 1989).

Persons living in developing countries have a higher likelihood of exposure to aflatoxins than would be expected in wealthy countries, and the dietary intake in those countries varies widely depending on level of affluence and/or aspects of culture. The level of protein typically provided to experimental animals, however, is optimum for growth and may not be comparable with the variations in diet seen among humans. This may lead to erroneous assumptions of risk to humans based on experimental data on animals without consideration of dietary effects.

Commercially available rodent diets typically contain 20–23.5% protein (Conner and Newberne, 1984), which is nearly twice the percentage (12%) recommended by the National Research Council (NRC, 1978) as adequate for growth and maintenance in rats. In previous studies (Woodall et al., 1996a and 1996b), we fed rats a semisynthetic diet with methionine-supplemented casein as the sole protein source and evaluated the ability of liver S9 fractions to activate various promutagens detectable in Salmonella strain TA98. We found that high (22%), adequate (12%), and nominally deficient (8%) levels of dietary casein affected the ability of Aroclor 1254 (Aroclor)- or 3-methylcholanthrene (3MC)-induced liver S9 to metabolize AFB (Woodall et al., 1996b). The S9s from rats induced by either Aroclor or 3MC and fed a dietary level of 12% casein were the most effective in metabolizing AFB to a mutagen.

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In the present study, we have extended these observations with Aroclor-induced S9 by performing mutagenicity assays (spiral Salmonella mutagenicity assays) using microsomes alone, cytosols alone, or S9s that had been reconstituted from combinations of microsomes and cytosols among the three dietary groups. We also examined whether adding exogenous bovine serum albumin (BSA) or glutathione (GSH) to the various hepatic microsomal preparations would affect their ability to activate AFB to a mutagen. BSA was used to test the hypothesis that an increase in protein levels would increase non-specific binding and, thereby, decrease mutagenic activity. GSH was added to test the hypothesis that GSH transferase activity may be involved in the dietary protein-related modulation in mutagenic activation and that increases in GSH would affect that activity.

We have also used inhibitors of both P450 and non-P450 enzyme systems associated with AFB metabolism to determine which are being affected by levels of dietary casein. A recent review (Massey et al., 1995) indicates that a wide array of P450 isoforms, including 1A1, 1A2, 2B1, 2B7, 2C1, 2C2, 3A3 and 3A4, are involved in the biotransformation of AFB to the reactive AFB-8,9-epoxide, the ultimate mutagen/carcinogen. In addition, several non-P450 enzyme systems are associated with AFB metabolism. These include cytosolic NADPH-dependent reductase, which is the major enzyme producing aflatoxicol, and two lipid hydroperoxide-dependent mechanisms that produce AFB-epoxide through microsomal prostaglandin H synthase and cytosolic lipoxygenases. Using the same hepatic preparations as in our previous study (Woodall et al., 1996b), we have now evaluated the effects of SKF-525A (SKF, an inhibitor of P450), indomethacin (ID, an inhibitor of prostaglandin H synthase), and nordihydroguaiaretic acid (NOR, an inhibitor of lipoxygenase and mitochondrial cytochrome reductase) (Zollner, 1990) on the ability of liver S9 fractions from rats fed different levels of dietary casein to metabolize AFB to a mutagen.

Most mechanistic studies are performed with microsomes or cytosols separately or with isolated enzymes. Mutagenicity studies, however, typically use S9, which contains both microsomal and cytosolic components. We performed mutagenicity assays with each component separately to try to test whether one or the other was responsible for the dietary protein-related differences in activation potential; neither was, and cytosol by itself did not activate AFB at all. We then attempted to reconstitute the activity of S9 by recombining these components, first to see whether the activation potential could be restored, and then to determine whether one component had the diet-related element.

These studies were performed near the completion of a series of experiments (Woodall et al., 1996a and 1996b) in an attempt to elucidate the phenomena observed in the initial studies. The test materials (S9s, microsomes and cytosols) were pooled within two groups of animals (yielding n = 2) by dietary regimen and inducer status in order to extend the usefulness of the material available. These limitations yield a set of qualitative results that may highlight areas for further investigation.

Materials and methods

**Experimental diets**

Three experimental diets were used as described previously (Woodall et al., 1996a and 1996b). These consisted of AIN-76A semisynthetic diets differing in protein content (methionine-supplemented casein), reflecting a high (22%), nutritionally adequate (12%) and nominally deficient (8%) level of protein for rats (NRC, 1978). The diets were made isocaloric by adjusting the amount of corn starch in the formulation. The composition of the 12% AIN-76A diet has been described previously (Butler and Dauterman, 1988). The diets were obtained from US Biochemical (Cleveland, OH).

**Chemicals**

AFB (1162-65-8), aflatoxin M1 (AFM, 6759-23-9), aflatoxin G1 (AFG, 1165-39-5), aflatoxin Q1 (AQF, 52819-96-2), aflatoxin (29613-03-8), SKF-525A (SKF, 6032-60-6), nordihydroguaiaretic acid (NOR, 500-38-9), indomethacin (ID, 53-86-1), GSH (70-18-8), β-nicotinamide adenine dinucleotide phosphate (NADP, 53-84-9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and glucose-6-phosphate (3671-99-6) were obtained from Sigma (St Louis, MO). Aflatoxin-dihydiol (AFB-Dih) and the AFB-glutathione conjugate (AFB-GSH) were kindly provided by Dr Thomas Harris of Vanderbilt University, Nashville, TN. All other chemicals were reagent grade and were acquired from Fisher Scientific (Fairlawn, NJ).

**Animals**

Details regarding animals and diets have been described previously (Woodall et al., 1996a and 1996b). Briefly, Fischer 344 male rats (60 days old, 120–150 g) were obtained from Charles River Laboratories (Raleigh, NC) and were allocated randomly to one of six large, raised-bed cages (three animals in each). Two cages were then allocated randomly to receive one of the three experimental diets, ad libitum, for a period of 6 weeks. One cage of rats at each dietary level served as the non-induced group and the second cage as the non-induced control. At the end of week 5, all animals in the Aroclor-induced group were given a single i.p. injection of Aroclor 1254 in corn oil (~500 mg/kg body wt) and were continued on their assigned diet; animals in the non-induced groups were injected with 0.25 ml of corn oil. At the end of week 6, all animals were fasted for 24 h prior to sacrifice but were provided with water ad libitum. The rats were killed by CO2 asphyxiation, final body weights were measured, and the livers were removed and weighed. The experiments described in this paper were performed using materials from two groups of induced rats treated by this protocol.

**Preparation of rat liver S9, microsomes and cytosol**

S9, cytosolic and microsomal fractions were prepared immediately from individual rats after livers were removed and liver wet weights determined. Liver S9s were prepared as described previously (Maron and Ames, 1983). Each liver was homogenized with buffer to create 50 ml of the liver–buffer homogenate; in our previous study (Woodall et al., 1996b), liver weights and liver/body weight ratios did not vary significantly between diets. One half of the volume of the S9 fractions was separated into several aliquots in 2 ml Nunc™ tubes, quick-frozen under liquid nitrogen, and held at −80°C until used in the mutagenicity assay; the second half was used to prepare cytosolic and microsomal fractions.

Microsomes and cytosols were prepared by centrifuging the S9s at 100 000 g for 1 h, quick-freezing the cytosols (supernatants) in liquid nitrogen, and storing them at −80°C. The microsomal pellets were resuspended in potassium phosphate buffer (50 mM, pH 7.5) and subjected to a second centrifugation at 100 000 g for 1 h. The final microsomal pellets were suspended in a potassium phosphate (50 mM) sucrose buffer (0.25 M); quick-frozen under liquid nitrogen; and stored at −80°C until use. Protein concentrations were measured for each S9, cytosol and microsomal preparation (Lowry et al., 1951) with BSA as the standard.

**Preparation of activation mixtures using S9s, microsomes, cytosols and reconstituted S9s**

Prior to use in a mutagenicity assay, activation mixtures were prepared containing a hepatic enzyme preparation (S9, microsomes, cytosol or reconstituted S9), cofactors (glucose-6-phosphate and NADP), and the MgCl2–KCl salt solution. Activation mixtures containing S9 or cytosolic protein were standardized to 5 mg/ml protein; however, those containing microsomes were standardized to 1 mg/ml protein to approximate the amount of microsomal protein present in S9. Reconstituted S9s were prepared by combining cytosols (5 mg/ml protein) with microsomes (1 mg/ml protein) and appropriate cofactors. The spiral Salmonella mutagenicity assay (spiral assay, Houk et al., 1988) was used to assess the activation characteristics of the S9s using Salmonella strain TA98, which was kindly provided by Dr Bruce N.Ames, University of California, Berkeley, CA. AFB was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 μg/ml. The bacteria, AFB and activation mixture (prepared with S9, microsomes or reconstituted S9) were deposited iteratively along the same spiral path. Both the AFB and the activation mixture were deposited at a logarithmically decreasing concentration from the center outward on a Vogel-Bonner minimal medium agar plate (10 cm) containing trace
histidine and excess biotin using a spiral plater (Spiral Biotech, Inc., Bethesda, MD).

The details of the spiral Salmonella assay have been described previously (Woodall et al., 1996a). Briefly, a 3-fold concentration of an overnight culture of Salmonella strain TA98 in Oxoid Nutrient Broth No. 2 was plated at a uniform deposition pattern on the plate along the entire track of the spiral. The AFB was plated next using a variable deposition pattern in which the highest concentration of AFB was deposited at the center-most spiral arm, with logarithmically lower doses deposited at each additional outward spiral. The activation mixture was then plated, also using a variable deposition pattern.

In assays performed with enzyme inhibitors, the inhibitor was plated using a uniform deposition pattern after AFB deposition and before deposition of the activation mixture. The activation mixture was then plated using a variable deposition pattern.

AFB was plated on triplicate plates for each activation mixture evaluated; also included were three matching negative control plates containing the bacteria, the activation mixture and DMSO. Revertant colonies were counted using a laser colony counter attached to a personal computer running the SALS software designed by Spiral Biotech for use with the spiral plater.

In vitro incubations

In vitro incubations were performed using S9s, microsomes and cytosols pooled by diet and inducer. Activation mixtures, prepared using the same protocol as used for the mutagenicity assays, were used in metabolic incubations performed with S9s and reconstituted S9s. The S9 activation mixtures (0.5 ml) were added to 1.0 ml of 0.1 M phosphate buffer, pH 7.4. Incubations performed with microsomes or cytosols contained 33 µl of either microsomes or cytosol in buffer with 10 µl of an NADPH-regenerating system (4.2 mM of NADP+, 14.1 mg glucose-6-phosphate, and 20 U glucose-6-phosphate dehydrogenase in 0.5 ml of buffer) to a final volume of 2 ml. Three tubes were prepared for each microsomal or cytosolic preparation tested: one tube was a zero-time control, and two tubes were replicates that were incubated for 20 min at 37°C in a shaking water bath. The reaction was initiated by the addition of 10 µl of a solution of AFB (1 mg AFB/1 ml methanol). The zero-time tubes contained 1 ml ice-cold methanol and were not incubated. After 20 min of incubation, the reactions were stopped by the addition of 1 ml ice-cold methanol. The samples were centrifuged in a table-top centrifuge for 5 min and then filtered through a 0.2 µm syringe filter into glass HPLC injection vials with Teflon-lined septa.

Analysis of metabolites by fluorescence HPLC

Analysis of the metabolites generated from in vitro incubations was accomplished by reverse-phase, solvent-gradient HPLC with fluorescence detection that was based on a previous method (Coles et al., 1985). A linear gradient of 25% to 100% methanol generated during 20 min with a 10 min hold at 100% methanol was produced by two Model 6000 pumps and a Model 680 gradient controller (Waters Corp., Milford, MA). The column was a Waters µ Bondapak™ C-18, 10 µ, 3.9 × 300 mm column; the flow rate was 1.0 ml/ min. The Model RF-551 detector was operated at high sensitivity with excitation at 365 nm and emission at 440 nm (Shimadzu Scientific Instruments, Inc., Columbia, MD). The column was equilibrated to initial conditions, and the baseline was automatically zeroed before each run. Data were collected and processed by a microcomputer running the EZ Chrom version 6.1 software (Scientific Software, Inc., San Ramon, CA) and equipped with Model SS 420 analog-digital converter boards.

Statistical analyses

Mutagenic potencies (slopes) were calculated from the linear portions of the mutagenicity dose–response curves for each plate using regression analysis. Analysis of variance (ANOVA) tests were performed using the resulting slope values to compare the effect of dietary casein levels on the ability of hepatic S9 to activate AFB. Post hoc, pair-wise comparisons between means were performed using least-square means to compare the results between diets to determine which dietary groups had characteristics that were significantly different from one another. Similar ANOVA comparisons and post hoc analyses were also applied to the peak areas measured in the fluorescence HPLC analyses. The SAS System software for the personal computer (Release 6.04, SAS System, Cary, NC) was used for all statistical analyses.

Results

Two types of experiments were conducted in this study: mutagenicity assays and in vitro incubations followed by fluorescence HPLC detection of metabolites. Mutagenicity assays were performed with pooled (by dietary group) microsomes, cytosols and reconstituted S9s to determine the activation potential of each fraction. Mutagenicity assays were also conducted with exogenous GSH or protein (BSA) to assess if variations in these components affected AFB activation. Additional mutagenicity assays with specific enzyme inhibitors were also performed to assess if inhibition of any one enzyme system eliminated the dietary protein-related effects. In vitro incubations with S9s, microsomes and cytosols were also conducted, and metabolites were detected by fluorescence HPLC. The results from the mutagenicity assays and in vitro incubation experiments are presented separately below.

Mutagenicity assays

The mutagenicity assays using S9s in this study are directly comparable with those reported previously for Aroclor-induced hepatic (A) S9s or (B) microsomes from the three dietary casein groups; (C) microsomes from the 22% casein diet group alone or with GSH or BSA.

Fig. 1. Dose–response curves from mutagenicity assays performed with Aroclor-induced hepatic (A) S9s or (B) microsomes from the three dietary casein groups; (C) microsomes from the 22% casein diet group alone or with GSH or BSA.
Table I. ANOVA analyses of mutagenic potencies (rev/µg) of AFB activated by S9, microsomes or reconstituted S9s

<table>
<thead>
<tr>
<th>Activation</th>
<th>Mutagenic potencies by diet&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANOVA results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8%</td>
<td>12%</td>
</tr>
<tr>
<td>S9</td>
<td>4.07&lt;sup&gt;1&lt;/sup&gt;</td>
<td>16.27&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>Microsomes</td>
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<td>0.65</td>
</tr>
<tr>
<td>Reconstituted S9s&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes from 8%</td>
<td>7.11</td>
<td>5.88</td>
</tr>
<tr>
<td>Microsomes from 12%</td>
<td>21.46</td>
<td>19.14</td>
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<tr>
<td>Microsomes from 22%</td>
<td>8.89</td>
<td>8.06</td>
</tr>
<tr>
<td>Cytosols from 8%</td>
<td>7.11&lt;sup&gt;1&lt;/sup&gt;</td>
<td>21.46&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytosols from 12%</td>
<td>5.88&lt;sup&gt;1&lt;/sup&gt;</td>
<td>19.14&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytosols from 22%</td>
<td>8.03&lt;sup&gt;1&lt;/sup&gt;</td>
<td>22.61&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mutagenicity data are the average of two independent experiments, each performed with triplicate plates. Different numeric superscripts denote significant differences in post hoc analyses of measurements between dietary groups (P < 0.05); the same numeric superscripts denote no significant difference.

<sup>b</sup>Significant at P < 0.05.

<sup>c</sup>Reconstituted S9s were made by combining the component in the first column with the complementary component from the dietary groups in the next three columns (i.e. microsomes with cytosols and cytosols with microsomes).

Table I shows the statistical analyses of the mutagenic potencies derived from Figures 1 and 2.

These studies show clearly that cytosol by itself did not metabolize AFB (based on in vitro incubations) nor did it activate AFB to mutagenic compounds (mutagenicity assays); microsomal fractions showed activation and metabolism of AFB, but did not show any dietary protein-related differences in those capabilities; only S9 and reconstituted S9 (microsomes + cytosol) showed activation and metabolism with dietary protein-related differences in those capabilities. Additionally, microsomes by themselves did not activate AFB to the same level as S9; however, when the same amount of microsomal protein (1 mg/ml) was combined with 5 mg/ml cytosolic protein, the activation potential was restored to the same level as seen with S9. This suggests the existence of a cooperative mechanism between microsomal and cytosolic components to produce the diet-related differences in mutagenic activation observed in native S9.

Comparisons of the mutagenic potencies from mutagenicity assays performed with inhibitors are shown in Figure 3. At low concentrations, SKF (a P450 inhibitor) reduced activation only slightly and did not alter the dietary casein effect (Figure 3A). However, at higher concentrations, activation was greatly inhibited across all dietary types, and the effect of dietary casein levels was eliminated. Similar results were produced by ID (a prostaglandin H synthase inhibitor) (Figure 3B). In contrast, NOR (a lipoxygenase and cytochrome reductase inhibitor) significantly increased activation at low levels, eliminated dietary casein level effects at 42 nM/plate equivalent and virtually eliminated all activation of AFB at 420 nM/plate equivalent (Figure 3C). NOR displayed no mutagenic activity in negative control plates. ANOVA analyses of the mutagenic potencies in the presence of inhibitors are presented in Table II.

In vitro AFB incubations and analysis of metabolites by fluorescence HPLC

Figure 4 shows examples of chromatograms of standards (Figure 4A) and the metabolites produced by incubations of AFB with either microsomes (Figure 4B) or S9 (Figure 4C) from the various dietary groups. Comparisons of the metabolism of AFB by S9s, microsomes and cytosols by peak areas for known and unknown metabolites are presented in Figure 5A, B and C, respectively. Variation in the absorbance and emission characteristics of AFB and its metabolites pre-
vents quantitative comparisons between the relative diminution of the AFB peak and the size of any of the metabolite peaks. However, comparisons between dietary casein levels for the same metabolite and between incubations are valid. Within these limits, identities for individual peaks were made by comparing relative retention times and peak shapes resulting from the incubations to those of pure compounds. Unidentified peaks were compared and identified by reference to their relative retention times on the column. Some low levels of contaminants were observed with most of the purified compounds used as reference materials, including the AFB parent compound, and some purified reference metabolite compounds (AFB-GSH and aflatoxicol) consistently gave multiple peaks (Figure 4A). ANOVA statistical analyses comparing the peak areas by dietary casein level for all major chromatogram peaks from metabolism with S9 are shown in Table III.

With regard to incubations using S9, the peak area for the AFB parent compound was lowest with the 12% casein diet, indicating that S9 from rats fed the 12% casein diet metabolized AFB most effectively (Figure 5A). Because metabolites of AFB, as opposed to the parent compound, are the mutagenic forms of AFB, this result is consistent with the results from the mutagenicity assays in which the mutagenic potency of AFB was greatest when activated by S9 from rats fed the 12% casein diet (Figure 1A). In addition, the levels of the metabolite AFM1 were greatest when AFB was incubated with S9s from rats fed the 12% diet (Figure 5A).

The appearance of two distinct peaks for AFB-GSH indicated that there were at least two metabolites of AFB being conjugated. One of these peaks (AFB-GSH1) was maximally produced by S9 from rats fed the 12% diet, which was the diet that produced the S9 that was most able to metabolize AFB to a mutagen. Thus, this diet produced both a high level of
Table II. ANOVA analyses of mutagenic potencies (rev/µg) of AFB activated by S9s in the presence or absence of inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration*</th>
<th>Mutagenic potencies by dietb</th>
<th>ANOVA results</th>
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<tr>
<td></td>
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<td>12%</td>
</tr>
<tr>
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<td></td>
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<td>7.24&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td>420.0</td>
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<td>ID</td>
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<td>NOR</td>
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<td>2.39&lt;sup&gt;1&lt;/sup&gt;</td>
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</tr>
</tbody>
</table>

*<sup>c</sup>*<sup>nM</sup>/plate equivalent.  
<sup>b</sup>Mutagenicity data are the average of two independent experiments, each performed with triplicate plates. Different numeric superscripts denote significant differences in post hoc analyses of measurements between dietary groups (<sup>P</sup>, 0.05); the same numeric superscripts denote no significant difference.  
<sup>c</sup>Significant at <sup>P</sup>, 0.05.

Fig. 5. Metabolite peak areas detected by fluorescence HPLC analysis from <em>in vitro</em> incubations of AFB with Aroclor-induced hepatic (A) S9s, (B) microsomes or (C) cytosols from rats from the three dietary casein groups.

mutagenic metabolites as well as a high level of conjugation (inactivation) of some metabolites.

With regard to incubations using microsomes (Figure 5B), no diet-related differences among the metabolites were found that were consistent with the mutagenesis data, which had shown no diet-related differences in the ability of microsomes to activate AFB to a mutagen (Figure 1B). Consistent with the mutagenesis data using microsomes in which no diet-related differences were found, the amount of AFM1 formed by microsomes also did not vary by diet (Figure 5B). However, the amounts of the second AFB-GSH metabolite and AFB-DhD generated by microsomes did increase with increasing levels of dietary casein (Figure 5B). With regard to incubations using cytosols (Figure 5C), no metabolism of AFB was evident from such incubations, which was consistent with the inability of cytosols alone to activate AFB to a mutagen (data not shown).

Discussion

The present study provides some insight into the mechanisms by which dietary casein levels affect the ability of hepatic S9 to activate AFB to a mutagen. Regardless of the dietary casein level, microsomes by themselves, but not cytosols by themselves, were able to activate AFB to a mutagen. However, the level of activation produced by microsomes was less than that produced by S9, and the diet-related differences in activation produced by S9 were not produced by microsomes alone. In contrast, diet-related differences were produced by microsomes when they were combined with cytosols, i.e. the enhanced ability of S9s from rats fed the 12% casein diet to activate AFB was also exhibited by reconstituted S9s composed of microsomes from the rats fed the 12% casein diet in combination with any cytosol, regardless of dietary group. Furthermore, the combination of cytosols with microsomes not only re-established the diet-related effect but resulted in reconstituted S9s with activation capabilities similar to those of the original S9s. These results suggest that dietary casein levels modulate a microsomal component that, in conjunction with cytosols, affects the ability of hepatic S9 to activate AFB to a mutagen.

Among the P450s, CYP1A1 has been demonstrated to activate AFB to the reactive ultimate mutagen AFB-8,9-epoxide (Massey <em>et al</em>., 1995; and Gonzalez, 1989). Although CYP1A1 also activates benzo[a]pyrene (BAP) to its mutagenic dihydriodil epoxide (Gonzalez, 1989), BAP activation by Aroclor-induced S9s was not affected by dietary casein levels in our previous study (Woodall <em>et al</em>., 1996b). Additionally, the ethoxyresorufin-O-deethylase activity in microsomes...
(mediated by CYP1A1) did not show any dietary protein-related differences (Woodall et al., 1996b). The isozymes in the 2B and 2C families that activate AFB are constitutive or phenobarbital-inducible; however, dietary casein levels did not affect AFB or BAP activation by S9s from phenobarbital-induced rats (Woodall et al., 1996b). Thus, neither CYP1A1 nor members of the 2B and 2C families of P450s are likely to have been affected by dietary casein in the present study.

In contrast, CYP1A2 has been associated with the production of both AFM1 and the AFB-8,9-epoxide from AFB but not of BAP-epoxide from BAP (Eaton et al., 1995). Evidence that this isozyme may be affected by dietary casein levels can be inferred from the HPLC data; the highest amount of AFM1 was produced by S9s from rats fed the 12% dietary casein level. This is consistent with the mutagenicity data, which showed that AFB was most mutagenic when metabolized by S9s from rats fed the 12% dietary casein level. A similar consistency between HPLC and mutagenicity data is seen for microsomes in which dietary casein levels did not affect either the activation of AFB to a mutagen or the production of AFM1 (Figures 1B and 5B).

AFM1 has ~3% of the mutagenic potency of AFB (Wong and Hsieh, 1976), and it is not likely that higher AFM1 production is directly responsible for the dietary casein level effects. Instead, the enhanced production of the AFB-8,9-epoxide likely accounts for the enhanced mutagenesis produced by S9s from rats fed the 12% dietary casein levels. AFB is metabolized to both the exo-8,9-epoxide and the endo-8,9-epoxide; however, only the exo-epoxide is mutagenic in Salmonella (Iyer et al., 1994). Chromatograms of GSH conjugates to both the exo- and endo-epoxides have been generated in incubations with recombinant CYP1A2, and the GSH–exo-8,9-epoxide conjugate has been found to elute first (Ueng et al., 1995). The first peak associated with AFB-GSH that eluted from HPLC in our in vitro incubations with S9 (AFB-GSH1 in Figure 5A) was highest when produced by S9s from rats fed the 12% dietary casein level versus the peak size for the 8% or 22% casein diets, which was also consistent with the mutagenicity results in which AFB was most potent when activated by the S9s from rats fed the 12% casein diet. Although we have not verified the identity of the two peaks (AFB-GSH1 and AFB-GSH2 in Figure 5A) associated with AFB-GSH as these two enantiomers, similar peaks and profiles were obtained in chromatograms of purified AFB-GSH (data not shown), and the order in which metabolites eluted from our HPLC system and that of Ueng et al. (1995) were identical for every other metabolite. Thus, the results from the mutagenicity, reconstituted S9 and metabolite studies suggest that CYP1A2 may be modulated by dietary casein levels, resulting in the enhanced ability of hepatic S9s from rats fed 12% dietary casein levels to metabolize AFB to a mutagen. Although CYP3A4 can also metabolize AFB to AFB-8,9-epoxide (Ueng et al., 1995), the ability of dietary casein levels to affect this isozyme cannot be inferred from the present study.

Retrospectively, western blot analyses were performed to determine the level of CYP1A2 and CYP3A4 present in the microsomal fractions; however, the samples showed signs of aging, no differences were observed between dietary casein levels, and no definitive conclusions could be drawn from the results. Specific P450 isozyme activities were probed in microsomes with the following assays: 7-ethoxresorufin-O-deethylase activity (CYP1A1); and benzphetamine-N-demethylase activity (CYP2B1 and CYP3A family). Cytochrome P450 levels were also measured in all microsomes, and GSH-transferase activities were measured in all cytosols. Inductions of rat liver enzymes were also performed with 3-methylcholanthrene (inducer for CYP1A1 and CYP1A2) and phenobarbital (inducer for CYP2B1 and CYP2B7); however, the mutagenicity assays did not show the same dietary protein-related effects with any of the S9s derived from those rats, indicating that the effect on those isozymes alone was probably not responsible for the diet-related effects.

The addition of BSA and/or GSH to the mutagenicity assays using TA98 had no effect on the mutagenicity of AFB (Figure 1C). However, a previous study (Jorgensen et al., 1987) found that such additions decreased the mutagenic potency of AFB in strain TA98 but had no effect in strain TA100. Using the SOS response as a genotoxic endpoint in Escherichia coli strains expressing human P450 3A4, researchers found that the genotoxic potency of AFB was stimulated by the addition of GSH (Ueng et al., 1995). The basis for these apparently conflicting observations is unknown; however, in our studies,
dietary casein levels produced no differences in GST or other phase II activities (Woodall et al., 1996a and 1996b).

Lipoxygenases are another cytosolic enzyme system that activates many compounds, and the use of the lipoxygenase inhibitor NOR might indicate whether this cytosolic enzyme system is also involved in the dietary protein-related effect. The results, however, were inconclusive, with increases in activation of AFB with low levels of NOR (without any diet-related effects), and increasing inhibition of activation with progressively higher levels of NOR, as shown in Figure 3 and Table II, NOR is also an inhibitor of NADPH cytochrome P450 reductase (Zollner, 1990), which may have affected the outcome more than inhibition of lipoxygenases.

The relevance of the present study to the effects of exposure of humans to aflatoxin is uncertain. CYP1A2 has been shown to be the most effective P450 isozyme at metabolizing AFB at low concentrations (Crespi et al., 1991), and the levels of AFB in most human dietary exposures are low (Eaton et al., 1994). In addition, CYP1A2 activity (phenotype) is highly variable among humans, showing a polymodal distribution (Eaton et al., 1995). Given that dietary protein levels vary considerably among populations and can influence toxicant metabolism (Anderson et al., 1982; Butler and Dauterman, 1988; Eaton et al., 1994; Klahr et al., 1994; Mallick, 1994), consideration of this factor may need to be included when evaluating the carcinogenic risk attendant to AFB exposure in populations (Guengerich, 1995; Strickland and Groopman, 1995).

In summary, this study has confirmed and helped elucidate the effects that dietary casein levels have on AFB activation, and it has added to our general knowledge of the metabolism of AFB by microsomes and S9s. The existence of a cooperative mechanism between microsomes and cytosols to produce the diet-related differences in the activation of AFB had not been noted previously in the literature. Additionally, the activities of phase I enzymes are often assayed in microsomal preparations alone, and it is rare for the microsomal metabolism to be compared with metabolism by S9. The observations on aflatoxin metabolism presented in our studies, showing an enhancement of microsomal metabolism by cytosol, may occur in the metabolism of other substrates as well.

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