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METABOLISM OF FUSARIN C BY RAT LIVER MICROSOMES

ROLE OF ESTERASE AND CYTOCHROME P-450 ENZYMES WITH RESPECT TO THE MUTAGENICITY OF FUSARIN C IN *SALMONELLA TYPHIMURIUM*

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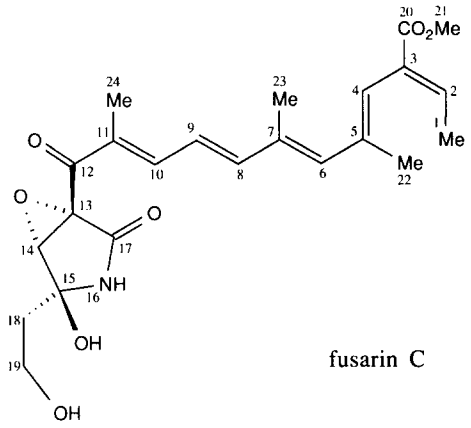
Abstract—Fusarin C (FC) is a potent mutagen present on *Fusarium moniliforme* contaminated corn. This compound requires metabolic activation for which microsomes from phenobarbital-induced rats are most effective. Inhibition of the simultaneously induced esterase activity, which produced a less mutagenic metabolite, doubled the mutagenicity of FC. Carbon monoxide inhibited the mutagenicity of FC, suggesting the involvement of a heme containing enzyme. However, monoclonal antibodies specific for the phenobarbital-induced cytochrome P-450 enzymes PB-4 and PB-5, while inhibiting O-demethylation of *p*-nitroanisole and aflatoxin B_1 mutagenicity, had no effect on FC mutagenicity. This implies that either these enzymes are not involved in the activation of FC or FC competes well with the antibodies for binding to the cytochrome P-450 enzymes. Two additional metabolites of FC were detected. One had an ultraviolet spectrum similar to FC; the other had a λ_{max} at 326 nm, and its retention time on reverse phase HPLC was very sensitive to changes in pH.

Epidemiological evidence strongly indicates that environmental agents, many of which are likely to be found in foods, are a major factor in human cancer induction [1]. *Fusarium moniliforme* is one of the most common fungi found on corn. In previous studies, it has been shown that papillomas and squamous-cell carcinomas can be induced in rats by feeding cornbread culture of *F. moniliforme* [2]. Based upon the distribution of *F. moniliforme* and the consumption of corn contaminated with this fungus, a possible association with human esophageal cancer has been suggested, especially in the areas of Linxian County, China, and Southern Africa [3–5]. During the past decade several compounds produced by this fungus were isolated, some of which are toxic and mutagenic. One of these, fusarin C (FC)¶, whose chemical structure has been elucidated [6, 7] (Fig. 1), was isolated from corn cultures of this fungus and was also found to occur naturally on corn in China, Southern Africa and North America [7–11]. Several studies have demonstrated that FC is toxic and mutagenic, and capable of inducing sister chromatid exchanges, micronuclei, chromosomal aberrations, and 6-thioguanine-resistant mutants in V79 cells [3, 4, 7, 9, 12]. We recently showed that FC increases the number of DNA breaks in bacteria in the presence of a metabolic activation system, induces asynchronous replication of the polyoma DNA sequence in H3 cells, a phenomenon also observed when these cells are exposed to a variety of other carcinogens, and alkylated *p*-nitrobenzyl pyridine in the absence of an added metabolic activation system [13].



fusarin C

Fig. 1. Structure of fusarin C.

Although FC contains an epoxide ring [6, 14], it still requires enzyme activation in order to obtain maximum mutagenic activity [4, 7, 12, 13]. The enzyme(s) responsible for the activation of FC was

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¶ Abbreviations: AFB_1, aflatoxin B_1; BCA, bicinchroninic acid; DIPFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; FC, fusarin C; MAb, monoclonal antibody; PM1, metabolite of FC lacking a 21-methyl ester group; NADP/H nicotinamide adenine dinucleotide phosphate/reduced; and PB, phenobarbital.

found to be a reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent microsomal system, for which phenobarbital (PB) was the most efficient inducer [12]. In addition to several cytochrome P-450 enzymes, other activities, such as esterases, are also greatly increased in the microsomes isolated from animals pretreated with PB [15, 16]. These enzymes may be responsible for the metabolic activation and/or deactivation of FC in the microsomes of PB-induced rats. In this study we examined the role of cytochrome P-450 systems and esterases in the metabolism of FC, by using monoclonal antibodies which inhibit specific cytochrome P-450 isoenzymes, carbon monoxide as a general inhibitor of the P-450 system, and diisopropyl fluorophosphate (DIPFP) as an esterase inhibitor. Further evidence was provided for the role of cytochrome P-450s in the activation of FC and of esterases in its inactivation.

METHODS

Chemicals. NADP/H, glucose-6-phosphate, diisopropyl fluorophosphate (DIPFP), *p*-nitrophenyl acetate, and aflatoxin B_1 (AFB_1) were obtained from the Sigma Chemical Co. (St Louis, MO). *p*-Nitroanisole, diazald and carbon monoxide (95%) were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Fusarin C was isolated from cornmeal inoculated with *F. moniliforme*, strain 07, as previously described [17].

Owing to the light sensitivity of FC, all experiments were performed in the dark or under yellow light. The isolated FC was further purified by HPLC (Zorbax C-18 reverse phase column, 250 mm × 4.6 mm, 60% methanol in water, 1 ml/min, retention time 21 min), and its structure and purity were confirned by its 500 MHz NMR spectrum. Monoclonal antibodies (Mab) 2-66-3, 2-8-1, 4-7-1, 4-29-5, 1-68-11 and 1-91-3 were prepared as previously described [18]. Liver microsomes, provided by Dr M. Miller, were prepared from BD IX rats induced with PB as previously described [13].

All spectrophotometric measurements were performed at room temperature in a Perkin Elmer Lambda 3B UV/VIS spectrophotometer except where indicated.

Assays for cytochrome P-450 and microsomal protein. The cytochrome P-450 concentration in microsomes from rats induced with PB was measured by the method of Omura and Sato [19]. In all enzyme assays, using MAb as inhibitor, the MAb concentration (mg/ml) was three times that of the cytochrome P-450. For controls, the protein contents were adjusted with MAb Hyhel-9, a monoclonal antibody with no specificity towards any cytochrome P-450. Protein concentrations in microsomes were determined with bicinchroninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard [20].

Esterase assay. Esterase activity in microsomes was measured using *p*-nitrophenyl acetate as substrate [21]. Briefly, 4 μg of microsomal protein was added to 0.1 M Tris–HCl buffer, pH 7.8, containing 5 μM *p*-nitrophenyl acetate. The final reaction volume was 1 ml. This mixture was incubated at room temperature for 10 min; then the absorbance of the product, *p*-nitrophenol, formed was measured at 400 nm against substrate and buffer as blank. A molar extinction coefficient for *p*-nitrophenol at pH 7.8 of 17,000 $M^{-1} cm^{-1}$ was used [21].

Inhibition of microsomal esterase by DIPFP was investigated using a similar assay. DIPFP, dissolved in dimethyl sulfoxide (DMSO) (10 μl), was added to reaction mixtures containing 4 μg protein but no substrate, at the indicated concentrations, and incubated at room temperature for 10 min. The substrate, *p*-nitrophenyl acetate, was added in ethanol (3.5 μl) to give a final concentration of 5 μM. The incubation was continued at room temperature for another 10 min after which absorbance was measured immediately at 400 nm.

O-Demethylase assay. *O*-Dealkylation activity in the microsomes was measured by direct spectrophotometry with *p*-nitroanisole as substrate [22]. To 200 μl of microsomes, diluted to a final concentration of 3 mg protein/ml, with standard S9 mix lacking NADP [23], was added 20 μl of 0.1 M *p*-nitroanisole in ethanol, and the reaction was started by the addition of 10 μl of 0.1 M NADP. After incubation at room temperature for 30 min, 500 μl of absolute ethanol was added to stop the reaction and precipitate the protein, followed by 30 μl of 5 N NaOH to raise the pH of the mixture. The above mixture was frozen in a dry ice–acetone bath for 2 min, and was then centrifuged to remove protein. The absorbance of the supernatant fraction was measured at 420 nm against a blank lacking NADP.

The effects of MAbs 2-66-3, 2-8-1, 4-7-1, 4-29-5, 1-68-11 and 1-91-3 on the activity of dealkylase in microsomes were determined. Microsomes (200 μl, 3 mg protein/ml) were added to 1.8 mg protein of anti-P-450 MAb or MAb-Hyhel-9, which is not directed against cytochrome P-450s, as a control, and incubated at room temperature for 20 min. Substrate and NADP were added, and the *O*-demethylase activity was measured as above. The inhibition of activity by MAbs was calculated as a percent of the control sample containing MAb-Hyhel-9 and samples containing no NADP as 100% inhibition.

Mutagenicity tests. All the mutagenicity assays used *Salmonella typhimurium* strain TA100 as previously described [23], with preincubation, except where noted.

Except where indicated, the S9 mix contained 0.1 M sodium phosphate, pH 7.4, 4 mM NADP, 5 mM glucose-6-phosphate, 33 mM KCl, 8 mM $MgCl_2$, and 0.3 mg microsomal protein/ml.

Diisopropyl fluorophosphate effects on the mutagenicity of FC. DIPFP was tested for cytotoxicity and effects on the mutagenicity of FC using the method of Schut *et al.* [24] with some modification. Before the addition of FC, 10-μl aliquots of DIPFP in DMSO were added to the S9 mix (0.5 ml) to yield final concentrations from 10^{-6} to 1 mM. These were gently vortexed and incubated at room temperature for 10 min. FC (928 pmol), or AFB_1 (32 pmol) as a positive control, and 0.1 ml of an overnight culture of *S. typhimurium* TA100 were added and incubated at 37° with shaking for 30 min. Top agar [23] (2 ml) was added to each tube, and the mixtures were poured onto minimal glucose agar plates. The revertants

were counted after incubation at 37° for 48 hr. The combined cytotoxicities of DIPFP and FC to the bacteria were tested by diluting 10 μl of the above incubation mixture with 0.1 M phosphate buffer, pH 7.4, to 2×10^3 bacteria/ml. The diluted bacteria (0.5 ml) and top agar (2 ml) were poured onto nutrient agar plates. Colonies formed after incubation at 37° for 36 hr were counted.

Influence of monoclonal antibodies on the mutagenicity of FC. The effects of MAbs on the microsomal enzyme activation of FC to mutagenic products were determined as previously reported [25] with some modifications. The MAb (1.8 mg/ml), in S9 mix (0.25 ml) lacking microsomes, was added to 0.25 ml of S9 mix (0.6 mg microsomal protein/ml) followed by 5 μl of 1 mM DIPFP. After mixing, the samples were incubated at room temperature for 20 min. FC (230 pmol) or AFB_1 (80 pmol) in DMSO (10 μl) and 0.1 ml of a TA100 overnight culture of *S. typhimurium* TA100 were added and incubated at 37° with shaking for 30 min. Top agar (2 ml) was added, and the mixture was poured onto minimal glucose agar plates. The revertants were counted after incubation at 37° for 48 hr.

Effects of carbon monoxide on the mutagenicity of FC. Carbon monoxide binds tightly to cytochrome P-450 enzymes and inhibits the activity of this system. The effect of carbon monoxide on the microsomal enzyme-mediated mutagenicity of FC was studied by using the Ames test [23]. S9 mix (without microsomes) (0.5 ml) and overnight cultures of *S. typhimurium* TA100 (0.1 ml) were bubbled gently with carbon monoxide or air, or allowed to stand for 5 min. DIPFP (6 μl, 1 mM) and 150 μg microsomal protein were added, mixed by vortexing, and incubated at room temperature for 10 min with the appropriate gas being gently blown over the surface of the sample. FC (230 pmol) was then added to each tube as indicated, and the tubes, sealed tightly with a Teflon-lined cap, were incubated at 37° with shaking for 30 min. Top agar (2 ml) was added and the mixtures were poured onto minimal glucose agar plates. The revertants were counted after 48 hr of incubation at 37°.

Isolation and identification of FC metabolite catalyzed by microsomal esterase. The FC esterase metabolite (PM1) which lacks the 21-methyl group of FC was isolated as previously reported [26]. Briefly 0.1 ml of microsomes from PB-induced rats (60 mg protein/ml) was suspended in 5 ml of 0.1 M phosphate buffer, pH 7.4, and incubated with 440 nmol FC at 37° for 2 hr. The incubation mixture was extracted twice with 10 ml chloroform, separating the phases by centrifugation. The aqueous layer, after removing residual chloroform with a stream of nitrogen, was applied to a C_{18} Sep-Pak cartridge (Water Associates), prewashed with methanol followed by water, and washed with 4×5 ml water. The metabolites were finally eluted with 3 ml methanol, and concentrated by rotary evaporation under vacuum.

The PM1 (70 nmol based upon its UV absorption and assuming the same molar extinction coefficient as FC [6], despite the slight change in λ_{max}), was dissolved in 100 μl methanol and cooled to 0°. Diazomethane in ether [27] (10 μl) was added and the solution stirred for 15 min. The reaction was stopped by evaporating the residual diazomethane with a stream of nitrogen. The reaction product(s) was dissolved in methanol and separated by HPLC on a Dupont 850 instrument. A Zorbax ODS reverse phase column (300 mm × 3.9 mm, Phenomenex Inc., Rancho Palos Verdes, CA) was used with a gradient from 40 to 100% methanol in water for 20 min and a flow rate of 1 ml/min. Absorption data were collected by LKB Bromma 2140 Rapid Spectral Detector from 220 to 370 nm.

Metabolites formed from [21-^{14}C]*fusarin C.* Microsomes from rats induced with PB (60 mg protein/ml, 0.2 ml) were suspended in 10 ml of S9 mix containing 10 μM DIPFP and incubated for 10 min before the addition of [21-^{14}C]FC [28] (235 nmol, 0.14 μCi), and the incubation was continued at 37° for 3 hr. The incubation mixture was then extracted three times with 10 ml ethyl acetate, separating the phases by centrifugation, and the ethyl acetate was removed by rotary evaporation under vacuum. The metabolites were separated by HPLC using a Zorbax ODS column with a gradient from 40% methanol in either 10 mM potassium phosphate buffer, pH 7.4, or 15 mM sodium acetate, pH 3.6, to 75% methanol in the same buffer over 40 min followed by a gradient to 100% methanol over 15 min.

RESULTS

Esterase activity of microsomes from phenobarbital-induced rats. Esterase activities of microsomal preparations are increased significantly by pretreatment of the animals with PB. The microsomal preparation used in these experiments had a specific activity of 1.2 μmol/min/mg protein using *p*-nitrophenyl acetate as substrate, which is similar to previously reported values for similar microsomal preparations [15]. Incubation of FC with microsomes, but without NADP/H, resulted in the characteristic yellow of FC no longer being chloroform extractable. Analysis of the metabolites, extracted from the aqueous phase using a C-18 Sep-Pak cartridge, by reverse phase HPLC showed that FC (19.5 min) was no longer present, but a new product was formed at 11.5 min. This compound had a UV spectrum similar to FC although the λ_{max} was shifted from 358 to 368 nm as expected after hydrolysis of the 21-methyl group. To confirm the structure of this compound, we reconverted it to FC by treatment with diazomethane which gave one product. It and FC had identical chromatographic and UV spectral properties.

Since this metabolite has been shown to be very weakly mutagenic compared to FC [26], we inhibited the microsomal esterase activity by DIPFP, which was tested from 10^{-6} to 1 mM (Fig. 2). Essentially complete inhibition of hydroylsis of *p*-nitrophenyl-acetate could be achieved at 1 μM DIPFP. The mutagenicity of FC in *S. typhimurium* TA100 was approximately doubled by pretreatment of the microsomes with DIPFP, with the optimum concentration being 1 μM (Fig. 2). Even at 1 mM DIPFP and 930 pmol FC, 92% survival of the bacteria was obtained. However, under these conditions the number of mutants declined from that seen at 1 μM

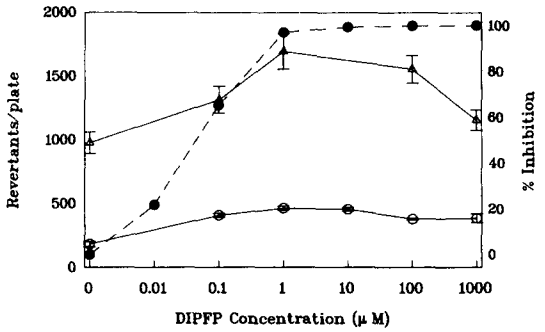

Fig. 2. Influence of diisopropyl fluorophosphate on microsomal carboxylesterase activity and mutagenicity of fusarin C in *S. typhimurium* TA100. Inhibition of microsome-catalyzed hydrolysis of *p*-nitrophenyl acetate by DIPFP (●). Incubation contained in 1 ml of 0.1 M Tris–HCl, pH 7.8, and 5 μM *p*-nitrophenyl acetate, 4 μg of microsomal protein and DIPFP at the concentrations indicated. After 10 min the *p*-nitrophenol formed was measured at 400 nm. The uninhibited preparations had a specific activity of 1.2 μmol/min/mg protein. Mutagenicity of fusarin C [(○) 120 or (△) 930 pmol/plate] was measured using *S. typhimurium* strain TA100 with metabolic activation using microsomes from PB-induced rats. Microsomes were preincubated with DIPFP at the indicated concentrations for 10 min prior to the addition of FC.

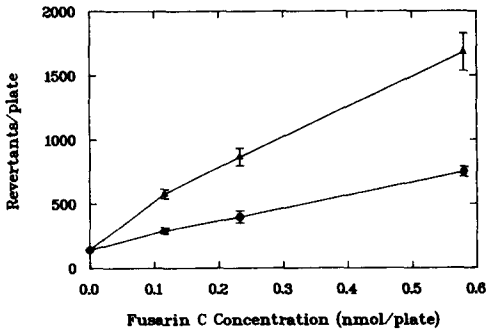

Fig. 3. Stimulation of the mutagenicity of fusarin C by diisopropyl fluorophosphate. The mutagenicity of fusarin C in the presence (▲) or absence (●) of 1 μM DIPFP was measured using *S. typhimurium* strain TA100 with metabolic activation using microsomes from rats induced with PB. Microsomes were preincubated with DIPFP for 10 min prior to the addition of FC.

DIPFP. Using 120 pmol FC, similar results were obtained, although the decline above 1 mM DIPFP was less pronounced (Fig. 2). The absolute number of mutants was, however, also lower. The approximately 2-fold stimulation of FC mutagenicity by 1 μM DIPFP remained fairly constant from 120 to 600 pmol FC/plate (Fig. 3). As expected, the mutagenicity of AFB_1 (32 pmol/plate) was unaffected by the absence or presence of 1 μM DIPFP (317 ± 29 and 325 ± 23 mutants/plate respectively).

Effects of carbon monoxide and monoclonal antibodies on the mutagenicity of fusarin C. Although the role of microsomes has been clearly shown to be critical for the mutagenicity of FC, which enzymes are involved has not. We tested carbon monoxide as a non-specific inhibitor of cytochrome P-450 oxidations and found (Table 1) that it reduced the mutagenicity of FC 3-fold. Some residual activity (about double background) was seen which was equivalent to that obtained when the FC was added immediately before pouring the reaction mixtures onto the agar plates (Table 1).

Table 1. Inhibition by carbon monoxide of fusarin C mutagenicity in *S. typhimurium* TA100

Experiment	Revertants/plate
A. Normal test	695 ± 36
B. Air preincubation	545 ± 43
C. CO preincubation	226 ± 25
D. FC added after preincubation	198 ± 14
E. Background	102 ± 12

To standard S9 mix (0.5 ml) was added 0.1 ml of a culture of *S. typhimurium* strain TA100. Samples were allowed to stand for 5 min, (Experiments A, D, and E), or were bubbled gently with air (B) or carbon monoxide (C). DIPFP (final concentration of 1 μM) and 150 μg of microsomal protein were added, and the samples were treated as before except that the gases were blown gently over the surface of the samples for 10 min rather than bubbled through. FC (230 pmol) was then added, except for experiments D and E, and each tube was sealed and incubated at 37° for 30 min. FC then was added to D and top agar (2 ml) to all samples, which were poured onto minimal glucose agar plates. The revertants were counted after 48 hr incubation at 37°.

MAb have been developed which inhibit cytochrome P-450 catalyzed reactions. Several of these were tested and inhibited oxidative demethylation of *p*-nitroanisole up to 52% (Table 2). One antibody showed 16% simulation of demethylation (Table 2). However, of these only one showed inhibition of microsome-induced mutagenicity in *S. typhimurium* TA100 by AFB_1, and none was effective with respect to FC.

Isolation of metabolites of [^{14}C]*fusarin C.* HPLC analysis of the microsomal metabolites showed, besides residual FC, that some PM1 was still formed, despite the presence of DIPFP in the incubations (Fig. 4), together with two additional radioactive metabolites (M2 and M3). The earlier eluting product (M2) had a spectrum similar to FC. The other (M3) eluted as a rather broad peak of variable retention time using conventional water–methanol mixtures. It eluted more sharply and with reproducible retention times which were very pH dependent if the solvents were buffered: 25 and 49 min at pH 7.4 and 3.6 respectively. This metabolite had a λ_{max} at 326 nm which, within the range used, was pH dependent.

DISCUSSION

Fusarin C, because of its multiple functional groups, could undergo a variety of enzymic reactions resulting in activation to mutagenic metabolites. Studies on these reactions have been difficult owing, in part, to the unavailability of radiolabeled FC. Any reactions involving the extended conjugated double bond system of FC would result in marked reductions

Table 2. Effect of monoclonal antibodies on the microsome-dependent mutagenicity of FC and AFB_1 in *S. typhimurium* TA100 and the inhibition of *O*-demethylase

Antibody	Specificity*	Aflatoxin B_1 (revertants/plate)	Fusarin C (revertants/plate)	*O*-Demethylase† (% inhibition)
Control‡		1418 ± 26	1142 ± 99	0
2-66-3	PB-4, PB-5	494 ± 37	1090 ± 105	52
2-8-1	PB-4, PB-5	1394 ± 20	1143 ± 33	25
4-7-1	PB-4, 3, PB-5	1210 ± 26	927 ± 160	35
4-29-5	PB-4, PB-5	1408 ± 40	1060 ± 23	40
1-68-11	2C, PB-1	1360 ± 17	1088 ± 62	16
1-91-3	P-450J	1356 ± 108	1132 ± 23	−16

Monoclonal antibodies, with the specifities shown, were tested for their inhibition of both the mutagenicity of fusarin C and aflatoxin B_1 in *S. typhimurium* TA100 and the oxidative demethylation of *p*-nitroanisole. Monoclonal antibodies (1.8 mg/ml) in S9 mix lacking microsomes (0.25 ml) and 0.25 ml of S9 mix containing 0.6 mg/ml microsomal protein were mixed, brought to 10 μM with respect to DIPFP, and incubated for 20 min at room temperature. FC (230 pmol) or AFB_1 (80 pmol) in 10 μl DMSO and 0.1 ml of an overnight culture of *S. typhimurium* TA100 were added. These mixtures were incubated at 37° for 20 min. Top agar (2 ml) was added to each tube, and the mixtures were poured onto minimal glucose agar plates. Revertants were counted after incubation at 37° for 48 hr.

* From Ref. 18.

† O-Demethylation was measured using *p*-nitroanisole as substrate. Microsomes (200 μl, 0.6 mg/ml protein) and antibody (200 μl, 1.8 mg/ml) were mixed and incubated at room tempeature for 20 min. Substrate (20 μl, 0.1 M *p*-nitroanisole in ethanol) and NADP (10 μl, 0.1 M) were added and incubated for 30 min at room temperature. Ethanol (500 μl) was added to stop the reaction and precipitate protein. The *p*-nitrophenol formed was converted to its anion by the addition of 320 μl of 5 M NaOH and, after centrifugation to remove precipitated protein, the absorption was measured at 420 nm. A negative value for inhibition indicates stimulation of metabolism.

‡ Control contained monoclonal antibody Hyhel-9 which does not recognize cytochrome P-450 proteins.

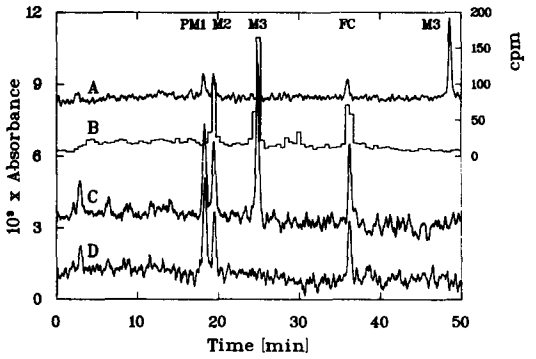

Fig. 4. Metabolism of [^{14}C]fusarin C by rat liver microsomes in the presence of diisopropyl fluorophosphate. Microsomes were preincubated with 10 μM diisopropyl fluorophosphate before the addition of fusarin C. The metabolites were extracted with ethyl acetate and separated by reverse phase HPLC using a 10 mM potassium phosphate buffer, pH 7.4/methanol gradient from 40% to 70% methanol in 40 min except for A in which the phosphate buffer was replaced by 15 mM sodium acetate, pH 3.6. Curves A and C show absorption at 330 nm, and D at 364 nm. The radioactivity data (B) are shown with their associated UV absorption (C and D).

in both the wavelength of maximum absorption and the molar extinction coefficient of the metabolite(s) making its detection, if formed in microsomal incubations, difficult.

We had noted that the characteristic yellow associated with FC was no longer extractable with chloroform after incubation of FC with microsomal preparations from PB-induced rats even when incubated in the absence of NADP/H. This observation was recently explained by the findings of Gelderblom *et al.* [26], who identified this metabolite of FC, designated PM1, as its free carboxylic acid derivative, formed by hydrolysis of the 21-methyl ester. Esterases are well known to be induced in the livers of rats treated with PB [15, 16]. We have confirmed this idenfication by re-esterification with diazomethane which converts PM1 back to FC. Since PM1 is about 100 times less mutagenic than FC [26], other metabolic pathways are probably involved in the activation of FC. We used DIPFP as an esterase inhibitor to prevent formation of this detoxification product. Complete inhibition of hydrolysis of *p*-nitrophenyl acetate was obtained at concentrations which were essentially neither toxic nor mutagenic to *S. typhimurium* TA100. As expected, preincubation of the microsomes with DIPFP increased the number of mutants observed in the bacteria. The reason for the decrease in the number of mutants from 1 μM to 1 mM DIPFP is not clear since, even at the highest concentration tested, 92% survival was obtained. It is possible that the DIPFP had some less specific inhibitory effects on other enzymes at these relatively high concentrations. Since about a 3-fold genetic variability in human populations with respect to esterase activity occurs [29], this may be an important variable with respect to the susceptibility of humans to the potential carcinogenicity of FC and the choice of animals for carcinogenicity studies.

Although microsomes and NADP/H are critical for activation to mutagenic metabolites, it is not known which specific enzymes are involved. Such

information would be helpful in searches for the critical metabolites involved in this activation. For these reasons we tested the effects of carbon monoxide as a non-specific inhibitor of cytochrome P-450 reactions, but one which should not affect other non-heme enzymes possibly involved in the activation of FC, and MAbs capable of inhibiting specific cytochrome P-450s. While not inhibitory to all reactions, carbon monoxide appears more general than some competitive substrates [30]. Bubbling air through the microsomes results in a slight inactivation of the system. The rates of bubbling, therefore, were carefully controlled to ensure the equivalence between the air and carbon monoxide experiments and, once microsomes were added, the gases were blown over the surface of the samples only. In the carbon monoxide experiment (Table 1, C), after subtraction of background (E), a 70% reduction in the mutagenicity was observed compared to experiment B. This reduction was equivalent to the effect of adding FC to the incubation mix immediately before pouring it onto the minimal glucose agar plates (D). The cytochrome P-450 enzymes are known to remain active in the top agar. The residual mutagenicity in the carbon monoxide experiments may have resulted from incomplete inhibition by carbon monoxide or recovery of the enzyme when re-equilibrated with air on the agar plate. These experiments provide strong evidence for the role of a heme protein in the activation of FC.

To gain more specific information on which cytochrome P-450s may be involved, we used MAbs. Unfortunately, from the experimental point of view, the different cytochrome P-450s show cross-reactivity with respect to substrate specificity and so, even if a particular form of P-450 is inhibited completely, it may not be reflected in total inhibition of a metabolic pathway for a particular substrate. We used the oxidative demethylation of *p*-nitroanisole as a general assay for PB-induced cytochrome P-450 activity (Table 2). The MAbs produced between 16% stimulation and 52% inhibition of oxidative demethylation. The stimulation of demethylase activity by MAb 1-91-3, which is specific for ethanol-induced cytochrome P-450, may have resulted from suppression of reactions other than demethylation, which in turn might have increased the yield of *p*-nitrophenol.

To determine the effects of this inhibition on mutagenicity in *S. typhimurium* TA100, we used aflatoxin B_1 as a positive control. Only MAb 2-66-3 inhibited the mutagenicity of AFB_1. The inhibition was about 65% which is similar to that previously reported [25]. However, none of the MAbs had any effect on the mutagenicity of FC, suggesting that either FC could effectively compete with the MAbs for binding to the cytochrome P-450 or that a minor form of the induced P-450s was involved in the metabolic activation of FC. The relative binding sites of the antibodies and the substrates tested (*p*-nitroanisole, AFB_1 and FC), or their affinities for the same sites on the cytochrome P-450s, may be sufficiently different to explain the difference in inhibition seen in these different assays. When the critical metabolite(s) of FC for its mutagenicity has been identified, it will be possible to measure the inhibition of metabolism and mutagenicity with the same substrate. One study [12] has demonstrated that, despite the increased esterase activities known to be present in microsomes from PB-induced rats, these microsomes were the most effective in activating FC to mutagens. A possible explanation for these results, and consistent with our observations using MAbs, is that a specific minor form of cytochrome P-450 induced by PB, but not Aroclor, is responsible for this activation.

The results from these experiments show that inhibition of esterase activity increases the mutagenicity of FC and that a carbon monoxide sensitive enzyme, probably a cytochrome P-450, is involved in at least one step of the metabolic activation of FC to mutagens. This cytochrome P-450, however, is unlikely to be the major form induced by PB since the mutagenicity of FC in *S. typhimurium* TA100, unlike aflatoxin B_1, is not sensitive to inhibition by MAb 2-66-3.

Initial experiments to identify metabolites formed in these microsomal incubations showed that two products, in addition to PM1, were formed. One was more polar than FC but had a similar UV spectrum. The other had a retention time which was very pH dependent, suggesting that under neutral conditions it was ionized. Its UV spectrum had also changed from that of FC to one having a maximum at 326 nm which was not affected by changes in pH from 3.6 to 7.4. This difference in UV spectrum would be predicted by reduction of the 12-carbonyl group to an alcohol. Alcohols generally eluted earlier by reverse phase HPLC than their corresponding ketones or aldehydes. This would not explain, however, the pH sensitivity of the HPLC retention time for which rearrangements of the λ-lactam ring are more probable [31]. No additional metabolites absorbing at shorter wavelengths were detected, demonstrating that the conjugated double bonds are relatively resistant to oxidation by the microsomal enzymes. Results from these studies, and on-going investigation of the metabolism of FC, will help in the design of animal carcinogenicity studies and the evaluation of human population concerning the possible role of FC in the etiology of esophageal cancer.

Note added in proof—Further studies on metabolite M3 have shown that it can be formed by nonenzymic reaction with reduced glutathione and there appears to be identical with compound A (Fig. 3, Gelderblom WCA, Thiel PG, van der Merwe KJ. The chemical and enzymic interaction of glutathione with the fungal metabolite, fusarin C, *Mutation Res* **199**: 207–214, 1988. Note: the 2–3 double bond is missing). Improved analysis of the UV spectra of M3 have shown a shift in wavelength from 323 nm at pH 7.4 to 333 nm at pH 3.6. Comparison of these maxima with that of FC and retanoic acid (342 nm, pH 7.4; 352 nm, pH 3.4) and retinal 373 nm are consistent with the assigned structure.

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