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METABOLIC ACTIVATION AND DEACTIVATION OF FUSARIN C, A MUTAGEN PRODUCED BY *FUSARIUM MONILIFORME*

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Abstract—The metabolic activation and deactivation of fusarin C, a mutagen produced by *Fusarium moniliforme* strain MRC 826, was studied by the *Salmonella typhimurium* mutagenicity assay using tester strain TA 100. A microsomal monooxygenase, preferably induced by phenobarbitone (PB) activates the mutagen to its active mutagenic form. Deactivation of the mutagenic metabolite seems to occur through chemical binding to thiol groups and by enzymatic conjugation mediated by a cytosolic glutathione-S-transferase.

A mutagenic compound, fusarin C, was isolated in our laboratory from a culture of *Fusarium moniliforme* (strain MRC 826) using the *Salmonella* microsome mutagenicity assay [1] as a monitoring system [2]. The chemical structure of fusarin C (Fig. 1) was determined [3] and it could be shown from NMR data that it was identical to the mutagenic compound isolated from a North American strain of *F. moniliforme* by Wiebe and Bjeldanes [4] who originally gave it the trivial name of fusarin C. During the isolation of the compound it was found that fusarin C was spontaneously converted to form two different compounds (P_1 and P_4) whereas exposure to longwave u.v. light gave rise to three other compounds (P_1', P_2' and P_4') [2]. Two other related compounds, fusarins A and D (Fig. 1) with similar u.v. absorption properties, were also found to be produced by the fungus [2, 3].

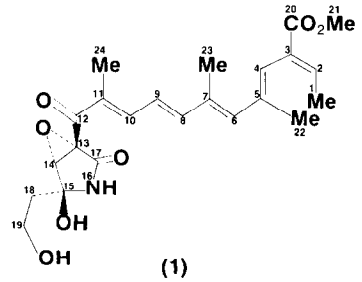
Fusarin C has to be activated by microsomal enzymes before it can bring about mutations in *Salmonella typhimurium* strain TA 100 [2]. This paper describes investigations into the mechanism of microsomal activation of fusarin C as well as studies on the inactivation of the reactive intermediate by conjugation reactions. The results are discussed in relation to the chemical structure of fusarin C and the structures and mutagenic activities of the related forms.

MATERIALS AND METHODS

Chemicals. NADPH, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were obtained from Boehringer Mannheim (SA) (Pty) Limited. Sodium phenobarbital (PB), glutathione (GSH) and cysteine were purchased from Merck (Darmstadt, FRG). The bacterial tester strain TA 100 was obtained from Dr B. Ames, University of California (Berkeley, CA), and Aroclor 1254 from Monsanto Company, St. Louis, MO.

Enzyme preparations. Induction of male BD IX rats with PB and Aroclor 1254 and the preparation of the liver homogenate fractions (S-9) were performed as previously described [1].

Microsomal and cytosolic fractions were prepared from the S-9 fraction obtained from PB induced male rats. The S-9 fraction was centrifuged at 13,000 *g* for 30 min and the supernatant was further centrifuged at 100,000 *g* for 60 min. The microsomal pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.4; 150 mM KCl) and stored at −70°. Prior to use it was centrifuged a second time at 100,000 *g* for



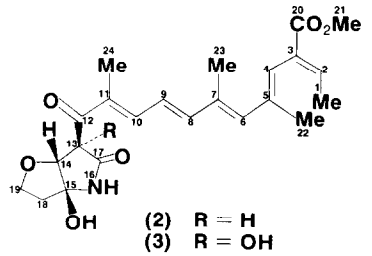


Fig. 1. Chemical structures of fusarins C (1), A (2) and D (3).

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60 min and resuspended in buffered 150 mM KCl to yield a microsomal preparation containing 5 mg protein/ml. The supernatant from the first centrifugation at 100,000 *g* was chromatographed on a Sephadex G-25 column (2.5 × 40 cm) using 50 mM sodium phosphate buffer (pH 7.4) as eluent to remove components of low mol. wt. The cytosolic proteins eluted in the void volume (approx. 65 ml). This cytosolic preparation containing 12 mg protein/ml was filtered through a sterile 0.45 μm 'Millipore' filter and stored at −70°. Protein concentrations were determined by the method of Lowry *et al.* [6] using bovine serum albumin as standard. Cytochrome P-450 concentrations were estimated from the dithionite-reduced difference spectra of carbon monoxide saturated samples using a molar extinction coefficient of 91 mM/cm [7].

Mutagenicity assay. The mutagenicity assay was performed as previously described [1]. Fusarin C was dissolved in DMSO and 0.1 ml vol were added per plate. Mutagenicity data were corrected for the background mutation rate (approx. 100 revertants/plate).

Effect of inducing agents. S-9 fractions from PB (41.2 mg protein/ml) and from Aroclor 1254 (39.2 mg protein/ml) induced rats were diluted with buffered 150 mM KCl to the same protein concentration as the uninduced control S-9 fraction (31.7 mg protein/ml). The activities of these S-9 fractions were compared in the plate incorporation assay at different protein levels (0.16, 0.79 and 1.89 mg protein/plate) and one concentration of fusarin C (1 μg/plate).

Mono-oxygenase dependent activation of fusarin C. The mono-oxygenase dependent activation of fusarin C (1 μg/plate) was studied in the plate incorporation assay substituting the S-9 fraction in the S-9 mixture with purified PB induced microsomes (720 μg protein/ml activating mixture). Six different activating mixtures were prepared. One contained the complete microsomal NADPH generating system while in the others, the microsomes, NADPH generating system, NADP, G-6-P and G-6-P dehydrogenase (1.75 units/ml) were omitted respectively and replaced by an equal volume of sterile water.

Conjugation reactions. The different conjugation reactions were studied indirectly by determining protection against the mutagenic effect of fusarin C. Standard activating mixtures containing PB induced microsomes (530 μg protein/ml), the NADPH generating system and 1.75 units of G-6-P dehydrogenase/ml were prepared. The protective effect of glutathione S-transferase was studied by using activating mixtures containing two different levels of cytosol (0.3 and 0.6 mg protein/plate) at three different levels of glutathione (0.25, 1.0 and 2.0 mM/plate). The protective effect of a thio group was studied using activating mixtures supplemented with different levels of cysteine (1, 2.5 and 5 mM/plate). Only one concentration of fusarin C (1 μg/plate) was used.

RESULTS AND DISCUSSION

Fusarin C has to be activated by rat liver microsomes to a reactive mutagenic form [2]. This activation was shown to be dependent on all components of the microsomal mono-oxygenase enzyme system (Table 1). Both the microsomal fraction and the reduced pyridine cofactor were indispensable for activation. The type of mono-oxygenase was further characterized by investigating the effect of different inducing agents (Fig. 2). Although the PB-induced S-9 fraction had a lower cytochrome concentration (cytochrome P-450) than that prepared from Aroclor 1254 (cytochrome P-450 and P-448) induced rats it brought about a higher mono-oxygenase dependent activation in the mutagenicity test. The activation of fusarin C seems therefore to be preferentially catalyzed by cytochrome P-450 for which PB is a very effective inducer [8].

Table 1. Mono-oxygenase-dependent-activation of fusarin C (1 μg/plate)

	Histidine revertants per plate*
Complete	1478
− Microsomes	2
− NADPH generating system	—
− NADP	15
− G-6-P	41
− G-6-P dehydrogenase	76

* Values represent mean of triplicate determinations. Spontaneous revertants were subtracted.

The effect of glutathione on the mutagenic activity of fusarin C in the absence and presence of different levels of cytosol is illustrated in Fig. 3. In the absence

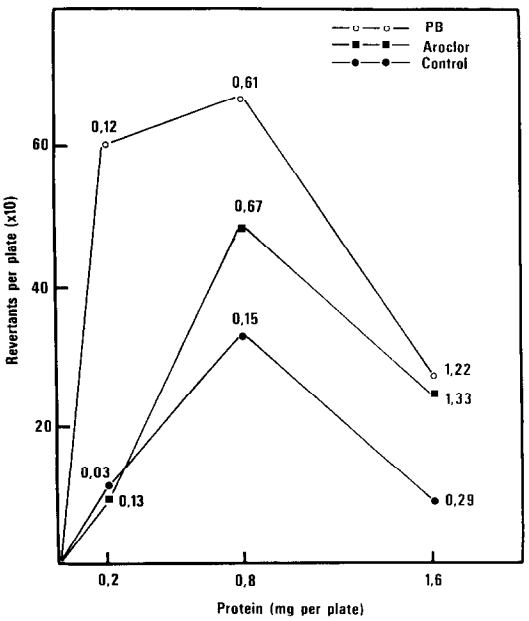

Fig. 2. Effect of induction of microsomal enzymes on activation of fusarin C. Three different S-9 levels of each S-9 fraction (0.16, 0.79 and 1.59 mg protein/plate) and one concentration of the mutagen (2 μg/plate) were used. The corresponding cytochrome P-450 concentrations (nmole/plate) are given on the dose–response curves for each S-9 fraction. Values are means of duplicate determinations.

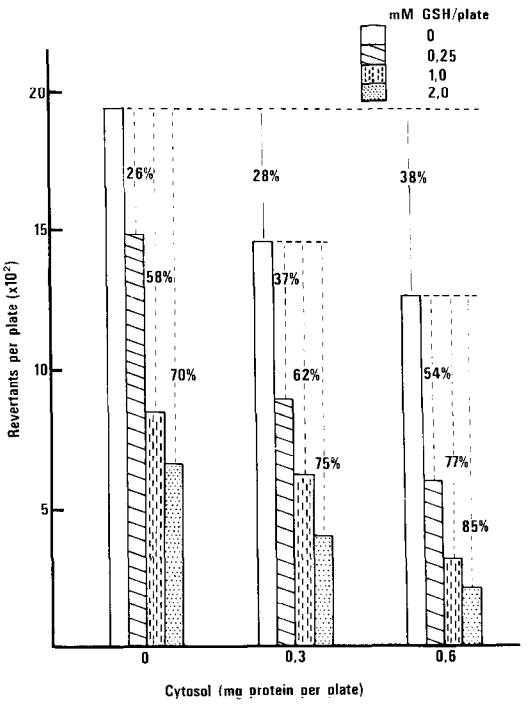

Fig. 3. Effect of cytosol and glutathione on mutagenicity (1 μg mutagen/plate). PB induced rat liver microsomes (270 μg protein/plate) were used and values represent means of triplicate determinations.

of cytosol) glutathione (2.0 mM) decreased the mutagenic response by 70%. This is probably brought about by a chemical binding of the activated intermediate to glutathione, as higher concentrations of another thiol, cysteine, also decreased mutagenesis (Table 2). In the presence of cytosol (0.6 mg protein/plate) the inhibitory effect of glutathione (0.25 mM/plate) could be enhanced from 26 to 54%, probably by conjugation of the activated intermediate with glutathione catalyzed by cytosolic glutathione-S-transferase. Recent studies showed that cysteine and glutathione were mutagenic against *Salmonella* strain TA 100 at levels of 6 mM and 8 mM per plate respectively [9]. In our experiments, however, the concentrations were too low to have any mutagenic effect.

Two other enzymes, the microsomal glucuronyl transferase and the cytosolic sulfotransferase seem to have no conjugating activity towards the mutagen as manifested by a protection against mutagenicity [10].

The chemical structure of fusarin C consists of a substituted 2-pyrrolidone moiety with a *2E*, *4E*, *6E*, *8E*, *10E* polyenic chromofore at position C_{13} (Fig. 1). The three u.v. induced forms (P_1', P_2' and P_4') do not lose their mutagenicity [2] and differ only from fusarin C in the side chain of the molecule (*8Z*, *6Z* and *10Z* stereoisomers respectively) [3]. In contrast to this, any alterations to the five membered ring lead to a loss of mutagenic properties. Fusarins

Table 2. The effect of increasing concentrations of cysteine on the mutagenic response (1 μg mutagen/plate)

Cysteine concentration (mM/plate)	Histidine revertants per plate*	% Inhibition or stimulation
0	1734	0
1.0	2076	+20
2.5	1617	− 7
5.0	1393	−20

* Values represent mean of triplicate determinations. Spontaneous revertants were subtracted.

A and D, the two natural occurring non-mutagenic compounds, do not contain the C_{13}–C_{14} epoxide. The spontaneously formed non-mutagenic forms (P_1 and P_4) still contain the epoxide but, in one case, it is displaced to position C_{14}–C_{15} by an anti-attack of the C_{15} hydroxy group on C_{14}, and in the other, the epoxide stays intact but the configuration of the groups at C_{15} is changed by epimerization [5].

It therefore seems that the C_{13}–C_{14} epoxide plays an important role in the mutagenic behaviour of fusarin C. The epoxide, with its electrophilic properties, could be the site for enzymatic conjugation with glutathione. It could also be responsible for the mutagenic properties of the molecule as binding to DNA can occur at this electrophilic site. The reason why fusarin C, already containing this epoxide, still requires activation to be mutagenic, is unknown. The isolation of the reactive mutagenic intermediate and the glutathione conjugates, which are in progress, could give answers to these questions.

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