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# The Mutagenicity of the Tyrosine Metabolite, Fumarylacetoacetate, Is Enhanced by Glutathione Depletion

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**The toxicity of tyrosine metabolites has been suggested, but not proven, to play a role in the ethiopathogenesis of hepatic alterations observed in hereditary tyrosinemia type I (HT I), a metabolic disease caused by a deficiency of the last enzyme in the tyrosine catabolic pathway, fumarylacetoacetate hydrolase. One of these metabolites, fumarylacetoacetate (FAA), is mutagenic in Chinese hamster V79 cells. We report here that FAA is a powerful glutathione depletor in this cell system. Moreover, the mutagenicity of FAA (100 $\mu$M) is potentiated by depletion of cellular glutathione (12% of control levels) by pretreatment with L-buthionine-(S,R)-sulphoximine. In this case, the mutation frequency induced by FAA is 10 times higher than in untreated, control cells. This enhancement is abolished by a partial replenishment of intracellular glutathione (32% of control levels) prior to FAA treatment. Reactive oxygen species are not generated during FAA treatment of glutathione-depleted or undepleted cells. Although the mechanism(s) underlying the mutagenic activity of FAA remains to be identified, these results show that the glutathione depletion activity of FAA may play an important role in the manifestation of its mutagenicity which likely contributes to the HT I-associated liver pathologies.** 

Hereditary tyrosinemia type 1 (HT I) is an autosomal recessive inherited disease caused by a deficiency of the last enzyme of the tyrosine catabolic pathway, fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2) (1-3). Progressive liver dysfunction is a representative feature of the acute form of the disease, while cirrhosis and hepatocellular carcinoma are often seen in the chronic form (4). Liver cancer has been observed in 37 percent of patients older than 2 years (5) and is responsible for 16 percent of deaths from HT I.

The toxicity of tyrosine metabolites accumulated immediately upstream of the block of the FAH hydrolytic step, fumarylacetoacetate (FAA) and maleylacetoacetate (MAA) (Fig. 1), has been suggested, but not yet demonstrated, to play a role in the ethiopathogenesis of the hepatic (and renal) alterations observed in HT I (1, 6, 7). This suggestion is based on the fact that FAA and MAA possess $\alpha,\beta$-unsaturated carbonyl compound structures that confer them electrophilic properties and potential biological activities. Alkylation of cellular macromolecules, such as DNA, and/or disruption of essential sulfhydryl reactions by complexation with, for example, proteins or glutathione (GSH), are among the suggested mechanisms underlying the toxic effects of FAA and MAA (1, 7). Given that liver cancer is not found in any other hereditary diseases affecting the tyrosine degradation pathway, it is unlikely that other tyrosine metabolites, e.g., homogentisic acid or p-hydroxyphenyl pyruvate, could be directly involved in its ethiology in HT I.

We recently reported that, among tyrosine metabolites that accumulate in HT I, i.e., FAA, MAA and succinylacetone (SA), only FAA (100 $\mu$M) displayed a mutagenic activity, using acquisition of 6-thioguanine resistance as a marker for the induction of gene mutations, in normal Chinese hamster V79 cells (8). The mutagenicity of FAA on these cells could only be ascribed as moderate, the mutation frequency induced by FAA being approximately 3 times greater than that observed in untreated cells. Since low levels of GSH have been encountered in the blood and liver of HT I patients (6,

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Abbreviations used: HT I, hereditary tyrosinemia type I; FAH, fumarylacetoacetate hydrolase; FAA, fumarylacetoacetate; MAA, maleylacetoacetate; SA, succinylacetone GSH, glutathione; GSSG, glutathione disulfide; DEM, diethylmaleate; BSO, L-buthionine-(S,R)-sulphoximine; HBSS, Hank's balanced salt solution; GSH-MEE, GSH monoethyl ester; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; ROS, reactive oxygen species; DCF-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein.

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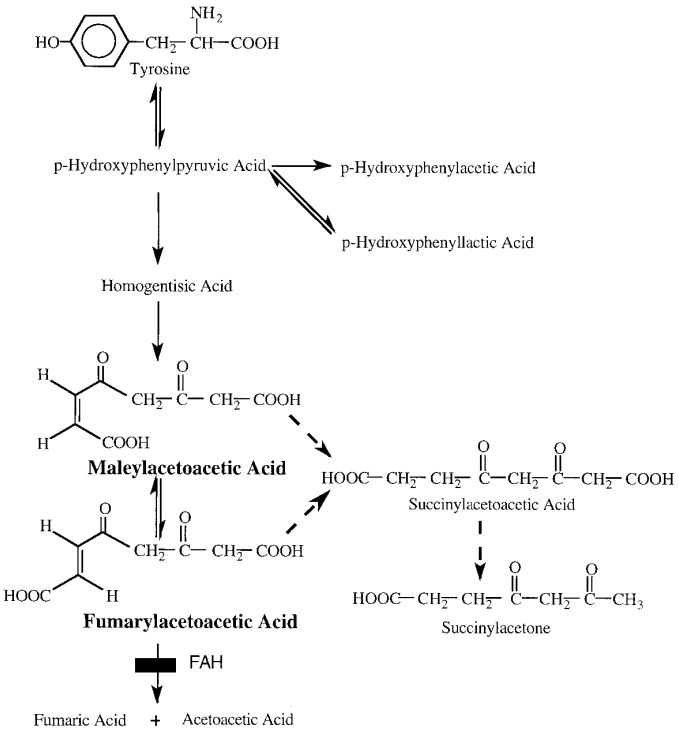


**FIG. 1.** Catabolic pathway of tyrosine. Abnormal metabolism occurring in HT I due to the block of FAH is shown by dashed arrows. The opening of the benzene ring of tyrosine occurs only during the oxidation of homogentisic acid and the metabolites MAA and FAA are the only possessing $\alpha,\beta$-unsaturated carbonyl compound structures.

9), we investigated the effects of variations in intracellular GSH levels on the mutagenic activity of FAA as well as the effects of FAA on GSH contents in Chinese hamster V79 cells. Here we show that FAA acts as a GSH depletor and that depletion of GSH potentiates the mutagenicity of this tyrosine metabolite.

## MATERIALS AND METHODS

*Reagents and cell culture.* FAA was synthetised as previously described (10) and kept frozen at −80°C until use. BSO, DEM and GSH-MEE were obtained from Sigma Chemical Co. (St. Louis, MO). Immediately before use, FAA, BSO or GSH-MEE were dissolved in HBSS (GIBCO BRL Products) supplemented with $NaHCO_3$ (0.35 g / L), pH 7.0. The concentration of the FAA solution was determined by spectral analysis (10) and adjusted to 1.5 mM with HBSS. All solutions were filter sterilized. Chinese hamster V79 cells were obtained from ATCC (N° CCL 93). In order to eliminate spontaneous $HGPRT^-$ mutants, the protocol of Bradley *et al.* (11) was used. Cells were grown in Dulbecco's modified Eagle medium (DMEM, high glucose; GIBCO BRL Products) supplemented with penicillin (100 U/ml)/ streptomycin (100 $\mu$g/ml)/amphotericin B (0.25 $\mu$g/ml) and 5% fetal bovine serum (FBS; Immunocorp Sciences Inc.), pH 7.4, at 37 °C with 5% $CO_2$. Trypsin (0.25% w/v)/EDTA (0.02% w/v), pH 7.8, was used to detach cells for doing sub-cultures.

*Treatment of cells and mutagenic assay.* Mutation frequency was measured on V79 cells as described by Glatt (12), with some minor modifications. Cells ($0.7 \times 10^6$) were seeded in 15-cm Petri dishes with 30 ml of normal medium (DMEM/FBS). For GSH depletion, BSO (0.2 or 1 mM, in medium) or DEM (0.5 mM, in HBSS) was added to cells after a culture period of 6 or 22 h, respectively. These treatments were terminated 18 h (BSO) or 2 h (DEM) later by changing the medium. For GSH replenishment, GSH-MEE (1 mM) was added to the BSO-containing medium 17 h after the beginning of this treatment and the medium was removed 2 h later. FAA treatment was done at concentrations of 35 or 100 $\mu$M. The FAA treatment was terminated 24 h later by changing the medium. After an additional normal culture period of 24 h, a first subculture of cells was done. The cell counts of the first subculture were used to evaluate the cytotoxicity of treatments. Then, $3 \times 10^6$ cells were seeded in one 15-cm Petri dish with 30 ml of medium. When low cell counts were encountered ($< 20 \times 10^4$ cells/ml) due to the high toxicity of a given treatment, the entire amount of cells (in 15 ml of medium) was seeded in a T-80 flask. Following a culture period of 48 h, cells were subcultured again at a density of $1.2 \times 10^4$ cells/cm$^2$. After 3 days (total expression time: 6 days), a third subculture was done. To determine the number of induced $HGPRT^-$ mutants, $1 \times 10^6$ cells were seeded in 15-cm Petri dishes (5 replicates/treatment) with 30 ml of medium containing the selective agent 6-thioguanine (7 $\mu$g/ml). The cloning efficiency was also determined by seeding 100 cells with 5 ml of normal medium in 6-cm Petri dishes (3 replicates/treatment). After 8 days (selection plates) or 10 days (cloning efficiency plates), the colonies were fixed, stained with methylene blue and counted.

*ROS measurement.* Intracellular ROS generation was measured using the fluorescent probe 2′,7′-dichlorofluorescein (DCF) (13). Briefly, cells were plated at a density of $2.3 \times 10^3$ per well in a 96-well tissue culture plate (Nunc). After a culture period of 6 h, BSO (0.2 mM) or the vehicle (HBSS) was added and cells were further incubated for 18 h. Then, medium was removed and wells were washed three times with HBSS. FAA (100 $\mu$M) was added in 0.1 ml HBSS containing 2′,7′-dichlorofluorescin diacetate (DCF-DA; 5 $\mu$g/ml) (Molecular Probes, Eugene, OR). The formation of the fluorescent derivative DCF, due to the oxidation of DCF-DA by ROS, was monitored by reading plates on a Cytofluor 2300 plate reader (Millipore, Bedford, MA), with an excitation wavelength of 485 nm and an emission wavelength of 530 nm, during a 2-h period.

*GSH, GSSG, and protein determinations.* Intracellular GSH and GSSG levels were measured on perchloric acid-deproteinized samples (14) as described by Suzukake *et al.* (15). Protein content was determined by the method of Lowry *et al.* (16).

## RESULTS

*FAA treatment causes an early decrease in intracellular GSH.* To study the effects of GSH depletion, two mechanistically different GSH-depletors were used, L-buthionine-(S,R)-sulphoximine (BSO) and diethylmaleate (DEM), the former specifically inhibiting GSH synthesis (17) and the latter adducting GSH (18). As shown in Fig. 2A (lanes 2 and 4, filled bar), intracellular GSH levels were reduced to almost the same extent (12-18% of control levels) by either BSO (0.2 mM × 18 h) or DEM (0.5 mM × 2 h) treatments. Twenty-four h after the end of the treatment and as expected from their mechanisms of GSH depletion, GSH levels are almost restored (79% of controls) in BSO-treated cells (Fig. 2A, lane 2, unfilled bar) while they increase (434 %) in DEM-treated cells (Fig. 2A, lane 4, unfilled bar). When cells were exposed to FAA (100 $\mu$M) in HBSS for 2 h, GSH levels were lowered to 5 % of control values (Fig. 2A, lane 5, filled bar). As after a DEM treatment,

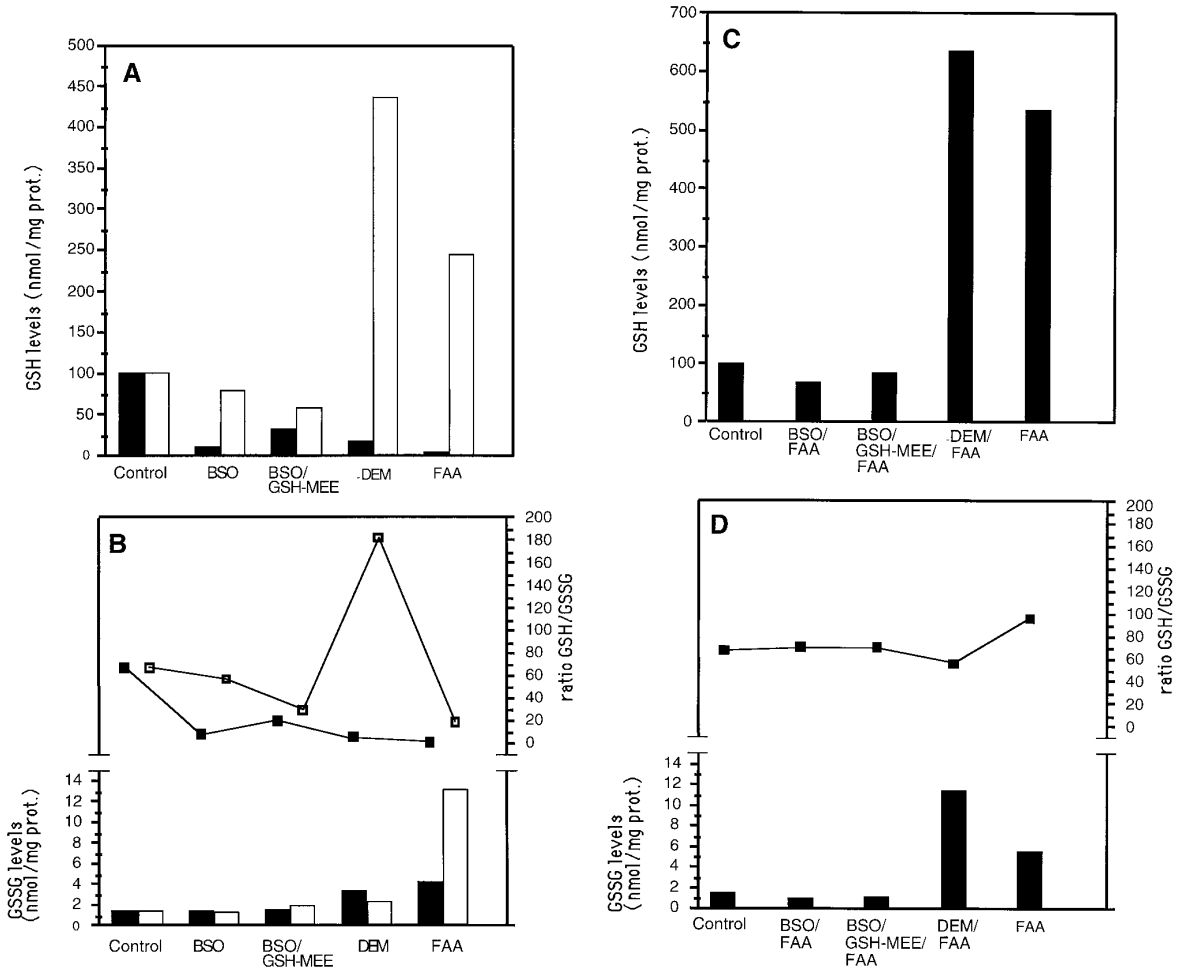


**FIG. 2.** Effects of FAA on intracellular GSH and GSSG levels following treatment of normal or GSH-depleted or replenished V79 cells. Intracellular GSH and GSSG levels were measured as described under Materials and Methods immediately after (filled bars) or 24 h after (unfilled bars) the end of the following treatments: (A, B) lane 1: untreated, control cells; lane 2: BSO (0.2 mM in medium × 18 h); lane 3: BSO (0.2 mM in medium × 17 h) followed by GSH-MEE (1 mM in medium × 2 h); lane 4: DEM (0.5 mM in HBSS × 2 h); and lane 5: FAA (0.1 mM in HBSS × 2 h). (C, D) Lane 1: untreated, control cells; lane 2: BSO (0.2 mM in medium × 18 h) followed by FAA (0.1 mM in medium × 24 h); lane 3: BSO (0.2 mM in medium × 17 h) plus GSH-MEE (1 mM in medium × 2 h) followed by FAA (0.1 mM in medium × 24 h); lane 4: DEM (0.5 mM in HBSS × 2 h) followed by FAA (0.1 mM in medium × 24 h); and lane 5: FAA (0.1 mM in medium × 24 h) after an initial 18 h-normal culture period. Before each treatment, cells were allow to growth for 6 h in normal medium, excepting when cells were treated with DEM or FAA in HBSS (× 2 h) where the initial normal culture period was 22 h. Values are the mean of two or three independent experiments, excepting control values that are the mean of all (8) control experiments (2 per treatment protocol); the SE for control values were less than 15 %. The ratio GSH/GSSG is shown in the upper side of charts B and D.

an overshoot of intracellular GSH levels (2-fold the control values) was observed 24 h following FAA removal (Fig. 2A, lane 5, unfilled). When DEM exposure of cells was followed by FAA treatment (in medium), the GSH levels went up 6 times the control values (Fig. 2C, lane 4). In contrast, GSH levels remained low following an FAA treatment of BSO-pretreated cells (Fig. 2C, lane 2).

Both DEM and FAA treatments (in HBSS, × 2 h) caused an increase in intracellular GSSG levels (Fig. 2B, lanes 4 and 5, filled bar), although the shift in the GSH/GSSG ratio was more pronounced for the FAA (5/4) than for the DEM (18/3) treatment. No changes in intracellular GSSG nor increases in extracellular GSH or GSSG levels were observed at time intervals shorter than 2 h of FAA treatment (data not shown). However, GSSG levels, as measured 24 h after the end of the treatment, tended to normalize in DEM-treated cells, but had additionally increased (by 3 times) in FAA-treated cells (Fig. 2B, lanes 4 and 5, unfilled bar). The exposure of normal cells to FAA added in medium (100 $\mu$M × 24 h) caused a 5-fold increase in GSH levels (Fig. 2C, lane 5) and, concomitantly, GSSG was almost 6 times greater than in controls (Fig. 2D, lane 5), making the GSH/GSSG ratio very similar to control values.

For GSH replenishment of BSO-treated cells, GSH

**TABLE I**

Frequency of HGPRT$^-$ Mutants Induced by FAA, MAA, or SA on Normal or Glutathione-Depleted V79 Cells

| Test compound | GSH depletion | GSH-MEE[b] exposure | Cell number[c] (% of control) | Cloning efficiency (%) | Mutant frequency $\times 10^{-6}$[d] |
|---|---|---|---|---|---|
| None[a] | – | – | 100 | 72 ± 11 | 6.7 ± 1.0 (5.7–8.1) (5)[e] |
| | + (0.2 mM BSO) | – | 91 ± 12 | 69 ± 15 | 9.3 ± 0.9 (7.9–10.8) (8) |
| | + (1.0 mM BSO) | – | 74 ± 11 | 62 ± 11 | 9.1 ± 3.4 (6.4–12.9) (3) |
| | + (0.5 mM DEM) | – | 106 ± 2 | 42 ± 10 | 10.9 ± 5.5 (4.8–15.6) (3) |
| FAA (35 $\mu$M) | – | – | 75 ± 9 | 69 ± 25 | 15.0 ± 4.2 (12.5–19.8) (3) |
| | + (0.2 mM BSO) | – | 44 ± 2 | 55 ± 22 | 15.7 ± 2.0 (13.5–17.1) (3) |
| | + (1.0 mM BSO) | – | 41 ± 4 | 72 ± 4 | 23.4 ± 2.0 (22.0–24.8) (3) |
| FAA (100 $\mu$M) | – | – | 57 ± 7 | 58 ± 12 | 22.0 ± 8.2 (13.8–31.6) (4) |
| | + (0.2 mM BSO) | – | 22 ± 7 | 41 ± 18 | 99.4 ± 29.6 (70.9–151.6) (7) |
| | + (0.2 mM BSO) | + (1.0 mM) | 22 ± 5 | 57 ± 8 | 34.0 ± 6.0 (28.6–40.0) (4) |
| | + (0.5 mM DEM) | – | 32 ± 1 | 58 ± 21 | 48.6 ± 18.6 (36.6–70.0) (3) |
| MAA (100 $\mu$M) | – | – | 84 ± 7 | 83 ± 22 | 7.8 ± 3.2 (5.6–10.2) (3) |
| | + (0.2 mM BSO) | – | 65 ± 8 | 74 ± 20 | 9.6 ± 3.4 (7.3–12.1) (3) |
| SA (700 $\mu$M) | – | – | 90 ± 25 | 88 ± 16 | 8.4 ± 1.1 (7.8–9.3) (3) |
| | + (0.2 mM BSO) | – | 68 ± 9 | 60 ± 16 | 11.2 ± 0.6 (10.8–11.7) (3) |

[a] Control cells were treated with the vehicle, HBSS.
[b] Exposures to BSO, DEM, GSH-MEE, or FAA were for 17–18, 2, 2, or 24 h, respectively, as described in details under Materials and Methods.
[c] Determined from the cell number present at the first subculture during the expression period.
[d] Determined after an expression and selection period of 6 days and 10 days, respectively. Five replicates ($1 \times 10^6$ cells/15-cm petri dish) were subjected to selection by 6-thioguanine (7 $\mu$g/ml), excepting for the BSO (0.2 mM)/FAA (100 $\mu$M) treated cells where only 2 or 3 replicates were done given the low cell count of those cultures.
[e] Values are the mean ± SE; the range of values and the number of single experiments are given in parentheses.

monoethyl ester (GSH-MEE), which rapidly crosses the plasma membrane and is hydrolyzed to GSH by intracellular esterases (19), was used. A partial replenishment of intracellular GSH (32 % of normal values) was achieved by exposing the BSO-treated cells to GSH-MEE (1 mM × 2 h) (Fig. 2A, lane 3, filled bar). When these cells were subsequently exposed to FAA (in medium, 100 $\mu$M × 24 h), GSH levels were 1.4 times greater than values observed on vehicle (medium)-treated cells but they did not exceed the corresponding control values (Fig. 2C, lane 3). No differences in GSSG levels were observed between BSO/GSH-MEE/FAA-treated cells and control cells (Fig. 2D, lanes 1 and 3).

*The mutagenicity of FAA is enhanced by previous GSH depletion of cells.* The mutation frequency induced by FAA (35 or 100 $\mu$M × 24 h), MAA (100 $\mu$M × 24 h) or SA (700 $\mu$M × 24 h) on normal or GSH-depleted cells is shown in Table I. Only FAA (100 $\mu$M) was mutagenic on normal cells, inducing a mutation frequency approximately 3 times higher than untreated cells a finding consistent with our previous report (8). However, when cells were depleted of GSH by BSO pretreatment (0.2 mM × 18 h), the mutation frequency induced by FAA (100 $\mu$M) increased almost 10 times over BSO-treated cells (99 vs 9 × $10^{-6}$). This effect is 5 times greater than the mutagenic effect of FAA alone on cells with normal GSH levels. GSH depletion of cells had no effect on the mutation frequency induced by MAA or SA which remained similar to untreated controls. Depletion of intracellular GSH (18 % of controls) by DEM also resulted in an increased mutagenic effect of FAA (100 $\mu$M), however this effect was only 2.5 times greater (49 vs 22 × $10^{-6}$) than the observed after FAA treatment of normal cells (Table I). Partial replenishment of intracellular GSH with GSH-MEE prior to FAA treatment decreased the mutation frequency induced by FAA (100 $\mu$M) to a value similar to that induced on cells with normal GSH levels.

By decreasing the FAA dose to 35 $\mu$M, the mutagenicity (and the cytotoxicity) of FAA on normal cells was decreased approximately 1.5 times compared to that observed with the 100 $\mu$M dose (Table I). Intracellular glutathione depletion by BSO (0.2 mM × 18 h) did not change the mutation frequency induced at the lower FAA dose. However, when the BSO dose was increased to 1 mM (GSH levels being decreased to 8 % of controls), the mutant frequency induced by FAA (35 $\mu$M) was increased 1.6-fold, reaching the value induced by the higher FAA dose (100 $\mu$M) on normal cells. It was not possible to study the combination of this high BSO dose with the high FAA dose (100 $\mu$M) because of cytotoxicity (data not shown).

*FAA treatment of cells does not induce the generation of ROS.* Since ROS, primarily hydrogen peroxide and hydroxyl radical, are potentially DNA damaging substances (20), we measured their intracellular generation with the fluorescent probe DCF in normal or GSH-depleted cells following FAA treatment . As shown in

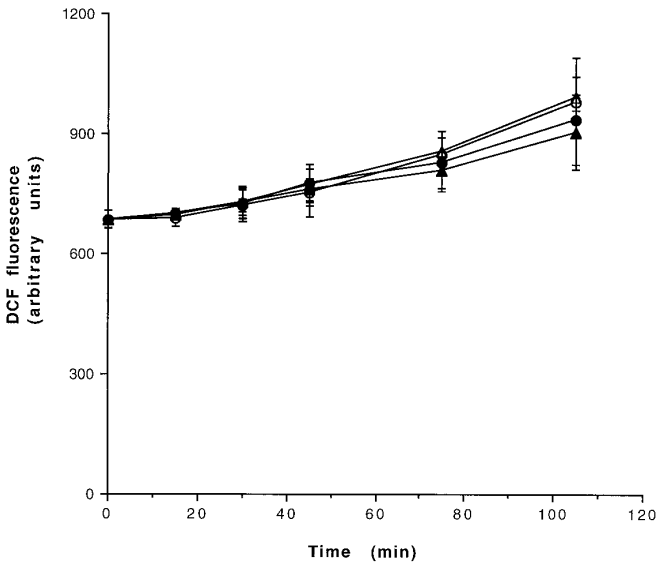


**FIG. 3.** Intracellular ROS generation in function of the time of incubation of normal or GSH-depleted cells with FAA. Normal cells + HBSS (open circles); BSO-pretreated cells + HBSS (open triangles); normal cells + FAA (solid circles); BSO-pretreated cells + FAA (solid triangles). Cells were incubated with FAA (or the vehicle, HBSS) plus DCF-DA and DCF fluorescence was measured for about 2 h. Values are the mean ± SE of three separate plates ; four wells per treatment protocol were measured in each plate and averaged.

Fig. 3, there were no major differences in DCF fluorescence levels between control (untreated) and FAA-treated cells during a 2-h period, regardless of the GSH status of the cells.

## DISCUSSION

Tyrosine metabolites have been suggested to be causative agents of the pathologies observed in HT I. Here we report some toxic effects of FAA in a mammalian cell system. We believed that these effects could play important roles in the ethiology of HT I-associated pathologies, specifically at the hepatic level. Firstly, FAA is a powerful GSH depletor in V79 cells. From the present data, the depletion of intracellular GSH caused by FAA seems to result mainly from the direct reaction of FAA with GSH, which is consistent with the formation of a conjugation product between FAA and GSH demonstrated by Edwards and Knox (21) in a cell-free system. This mechanism of GSH depletion, similar to that of DEM (22), is also confirmed by the late overshoot in intracellular GSH contents that follows the initial FAA-induced GSH decrease. The fact that the overshoot on GSH levels induced by FAA was potentiated by pretreatment of cells with DEM but was not detected on BSO-pretreated cells suggests that it results from increased GSH resynthesis. This event could also account for the relatively high levels of intracellular GSSG observed after the end of the FAA treatment of cells not pretreated with BSO, excess resynthetised GSH being oxidized to GSSG via the normal activity of glutathione peroxydase. The other substrates of this enzyme, ROS, do not seem to contribute to the GSSG increase in FAA-treated cells since their production was not induced in these cells (or in BSO-pretreated cells) during the first two hours of incubation with FAA. In the case of DEM-treated cells, oxidation of excess GSH to GSSG did not appear to function efficiently and may be ascribed to a nonspecific sulfhydryl reagent effect of DEM at the glutathione peroxydase level (23). Whether the late accumulation of intracellular GSSG observed in FAA-treated cells results from an impairment effect of FAA at the glutathione reductase level or a decrease in the NADPH/$NADP^+$ redox level remains to be investigated.

Secondly, besides its intrinsic GSH depletion activity, FAA is mutagenic in V79 cells (8). What is a noteworthy finding of the present study is that the mutagenicity of FAA is modulated by the net GSH content of cells. This is evident from the observation that GSH depletion of cells prior to FAA treatment enhanced the mutagenic effect of FAA, with the BSO pretreatment of cells providing the greatest enhancement. Given that the main difference between BSO and DEM treatments was the extent of the post-treatment period in which intracellular GSH levels remained low, it is clear that a true sustained GSH-depleted status of cells, such as induced by BSO, will favor the occurrence of the molecular events mediating the mutagenic activity of FAA and will potentiate this activity. The buffering action of intracellular GSH on the mutagenicity of FAA is also suggested by two other observations: i) partial GSH replenishment of cells (32 % of controls), through GSH-MEE exposure is sufficient to prevent the enhanced mutagenicity of FAA (100 $\mu$M) on BSO-treated cells, and ii) even a lower FAA dose (35 $\mu$M) becomes mutagenic when GSH levels are sufficiently reduced (8 % of controls; 1 mM BSO used). In the latter case, the observed mutagenic effect is equivalent to the one induced by a dose of 100 $\mu$M FAA. Thus, it seems that when large amounts of FAA suddenly occur in a cell having a normal GSH content, a fraction of FAA will complex with GSH (and likely also with protein thiols) and free FAA may then exert its mutagenic activity. A chronic depletion of cellular GSH caused, for example, by the continuous and abnormal intracellular production of FAA, will thus potentiate the mutagenic activity of free FAA. This is consistent with the stronger mutagenic effect of the higher FAA (100 $\mu$M) dose used when GSH synthesis is blocked by BSO. A similar buffering activity of intracellular GSH has been observed towards the hepatotoxic effect of acetaminophen. Acetaminophen-induced necrosis, caused by the covalent binding of a reactive interme-

diate to vital cell macromolecules, only occurs when large doses of the drug are used or when GSH contents of the cell are sufficiently decreased prior to drug exposure by a compound such as DEM (24).

Mutations at the HGPRT locus in cells exposed to chemical agents could arise from a large variety of DNA lesions (25). The involvement of ROS in the mutagenicity of FAA can be ruled out since incubation of normal or BSO-treated cells with FAA did not induce their production. The direct alkylation of DNA by FAA must be considered as one likely mechanism for the induction of such mutations. The difference between the intrinsic mutagenic activities of FAA and MAA could be merely due to steric factors determining, for example, the efficacy of the enzymatic repair of the promutagenic DNA lesion. Another possibility is that FAA by itself, directly or indirectly, could impair the enzymatic repair of its induced DNA lesion. For example, the thiol-depleting agent dimethyl fumarate, which has a structure similar to FAA and depletes GSH (and protein thiols) by covalent bond formation as DEM, has been reported to sensitize aerobic V79 cells to irradiation by altering enzymatic (thiol depending) DNA repair processes (26). In line with this, reaction of FAA with thiol groups of a human serum protein such as albumin has been reported (27). Interestingly, as it generally occurs in diseases associated with abnormal DNA repair, an increased chromosomal damage has been observed in cultured fibroblasts from a HT I patient (28). Moreover, human cells having low GSH levels due to a genetic deficiency in GSH synthetase have a reduced capacity to repair irradiation-induced DNA damage (29). In one patient suffering from GSH synthetase-deficiency, Lloyd *et al.* (27) have also observed a concomitant deficiency of FAH, and suggested that GSH is essential for maintaining the activity of this enzyme and could be a mediator in the pathogenesis of symptoms in this genetic disorder and in HT I. From these observations and from our data, we propose that, in the liver of HT I patients, a fraction of overproduced FAA reacts with GSH (and protein thiols) which causes both an impaired enzymatic DNA repair and a decrease of residual FAH activity (if any). The latter, the low GSH content and the low conjugation capacity (due to an insufficient rate of GSH regeneration) of hepatocytes contribute to the further accumulation of FAA. The free, unconjugated fraction of FAA might then react with DNA where mutation(s) would be favored by the impaired repair of the FAA-induced DNA damage.

In summary, we provide here, for the first time, a direct evidence for the mutagenic activity of FAA in a mammalian cell system and for the modulating effects of intracellular GSH on this activity. We believe that FAA, as the only natural mutagen occurring in the tyrosine catabolic pathway, must be directly involved in the initiating step of the carcinogenic process occurring in the liver of HT I patients. Low hepatic GSH contents, that result from the potent GSH depletion activity of FAA, must be considered as a determinant contributing factor to this process.

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