Some mass-spectral and n.m.r. analytical studies of a glutathione conjugate of aflatoxin B₁

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A system for the formation of an aflatoxin B₁-reduced glutathione conjugate in vitro was developed, capable of yielding 80% conversion of aflatoxin B₁ into the conjugate. A reverse-phase high-pressure-liquid-chromatography system was also devised that not only facilitates improved resolution of the compound but that, by manipulation of the pH, is also capable of an extensive purification of the compound from other aflatoxin B₁ metabolites in a single step. Material produced by these techniques, after further purification, has been used in ¹H-n.m.r. and mass-spectroscopic studies. Results were obtained that support the proposed linkage of the aflatoxin B₁ to reduced glutathione in a 1:1 molar ratio via a thioether linkage. Amino acid analyses were also consistent with this structure. The absence of a Schiff-base linkage of aflatoxin B₁, 8,9-dihydrodiol to glutamate was further demonstrated by the presence of a γ-glutamyltransferase-catalysed-transferable glutamate moiety. These data are consistent with the structure 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-aflatoxin B₁.

In common with many toxic xenobiotics, the hepatocarcinogenic mycotoxin AFB₁ requires metabolic activation in order to bind covalently to macromolecules in the target organ (Garner et al., 1972). It is probable that macromolecular binding is the biochemical mechanism responsible for both the acute and the chronic toxicities in susceptible species, which include the rat (Campbell & Hayes, 1976; Campbell et al., 1978; Ueno et al., 1980). Strong evidence has supported the hypothesis that AFB₁ is activated by conversion into an epoxide (Garner et al., 1972; Swenson et al., 1977; Garner et al., 1979). A protective action may be afforded by reaction of this electrophilic AFB₁ metabolite with GSH (Lotlikar et al., 1980; Neal et al., 1981a). The presence of an AFB₁-GSH conjugate in the bile of AFB₁-treated rats, and its formation in vitro in liver-derived subcellular fractions, has been reported (Degen & Neumann, 1978, 1981). In those studies, however, insufficient material was available for ¹H-n.m.r. and mass-spectral studies. In view of the considerable interest in the possible involvement of aflatoxin in human disease, and the probable significance of the formation of an AFB₁-GSH conjugate in this process, it appeared desirable to examine the structure of the conjugate in more detail. We have therefore sought a more efficient system for the production of this material, and have utilized this for ¹H-n.m.r. and mass-spectroscopic studies. The present paper deals with the results of these.

Materials and methods

Chemicals

AFB₁ was obtained from Makor Chemicals, Jerusalem, Israel, and [¹⁴C]AFB₁ (specific radioactivity 120mCi/mmol) from Moravek Biochemicals, City of Industry, CA, U.S.A. Compound AT125 [L-(αS,βS)-2-amino-2-(3-chloro-4,5-dihydro-isoxazol-5-yl)acetic acid] was a gift generously provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.

Animals and preparation of subcellular fractions

Microsomal fractions, suspended in 150 mM-KCl, were prepared from the freshly excised livers of adult phenobarbitone-prestimulated male Fischer F344 rats (0.1% phenobarbitone in drinking water for 5 days), adult male C57 BL/10 SCSN mice and adult
Rhode Island Red × Light Sussex hens (Neal et al., 1979; Neal & Colley, 1979a; Neal et al., 1981b). The corresponding cytosol fractions were prepared simultaneously and retained. All fractions were stored at −70°C until required.

**Incubations in vitro**

All procedures were conducted under subdued light to avoid photochemical decomposition of AFB1. Incubations were performed with the necessary cofactors, potassium phosphate buffer, pH 7.4, 5 mM GSH (neutralized) and 128 nmol of AFB1 in 20 μl of dimethyl sulphoxide as previously detailed (Neal & Colley, 1979b; Neal et al., 1979; Metcalfe et al., 1981). When samples of conjugate were prepared for n.m.r. and mass-spectral analysis, 12 replicate incubations were used (total AFB1, 480 μg). [14C]AFB1 was used in these incubations (specific radioactivity approx. 1 mCi/mmol).

**H.p.l.c. and other assays**

Samples, precipitated with methanol, were clarified by centrifugation at 1000 g, and the methanol was evaporated from the supernatant under a stream of N2 at 35°C. Samples were then freeze-dried and stored at −70°C until required. For h.p.l.c. they were reconstituted in methanol/water (1:1, v/v) and centrifuged for 60 min at −25°C, and the supernatant was adjusted to pH 4.0 or 7.0 and centrifuged at 1500 g for 30 min at −10°C, and the supernatant obtained further clarified by passage through glass-fibre filters (GF/A, followed by GF/F; Whatman Labsales, Maidstone, Kent, U.K.).

Samples were chromatographed on a laboratory-packed 100 mm × 4.6 mm h.p.l.c. column of Magnosphere 5 μ ODS (Magnus Scientific Instrumentation, Aylesbury, Bucks., U.K.) after passage through a guard column (65 mm × 1.2 mm) of Co Pell ODS. Routine elution was by means of a gradient from methanol/water (3:17, v/v) to methanol/water (2:3, v/v), 0.01% H3PO4. Separations were greatly improved by the inclusion of 8% (v/v) acetonitrile in all solvents. Radioactivity in h.p.l.c. fractions was determined in a liquid-scintillation spectrometer after addition of Picofluor 30 scintillator (Packard Instruments, Caversham, U.K.). Samples to be used for n.m.r. and mass spectrometry were specially purified by solvent extraction, ion-exchange chromatography (DE-52 DEAE-cellulose, Whatman) and gel filtration chromatography (Sephadex G-15 followed by Sephadex LH20), before h.p.l.c. by modifications of the procedures used by Degen & Neumann (1978) and Irving et al. (1967). Overall yields after these purification procedures in terms of recovery of AFB1 present in the conjugate after the initial microsomal incubations were approx. 25%. T.l.c. was performed on Silica gel G plates and developed with butanol/acetic acid/water (2:1:1, by vol.).

Protein was assayed by the method of Lowry et al. (1951), with bovine serum albumin as standard.

**N.m.r. and mass spectrometry**

The 360 MHz 1H-n.m.r. spectrum was obtained with approx. 0.2 μmol of conjugate dissolved in 99.98 H2O (40 μl) by using a Bruker model WH 360 spectrometer operating in the Fourier-transform mode and equipped with a 2.5 mm micro-probe. The 100 MHz spectra were obtained on a JEOL MH100 instrument. Field-desorption mass spectrometry was performed with a Finnigan MAT 312 spectrometer/SS 188 data system equipped with a combined FD/FI/EI ion source and a precisely adjustable direct-insertion probe (Przybylski et al., 1982). Activated-carbon field ion emitters with average needle lengths of 30–50 μm were prepared by the high-temperature activation procedure (Schulten & Beckey, 1972). Samples (1–2 μl) of solutions of approx. 0.5 μg/μl were loaded on the emitter by the syringe technique, with the use of a three-dimensional micro-manipulator (Przybylski et al., 1982). Methanol/water (approx. 1:1, v/v) was used as solvent. The sample was heated with an emitter heating-current programmer (Linden, Bremen, W. Germany) at a linear rate of 4 mA/min, and continuous spectra were obtained between 15 and approx. 20 mA by the data system. The spectrum acquired by accumulation of 20 scans (16–21 mA) by the data system was evaluated for molecular and fragment ion analysis (Przybylski, 1982). Other instrumental conditions were as previously described (Przybylski, 1982).

**Amino acid analysis**

The proposed AFB1-GSH conjugate (approx. 3 nmol) was hydrolysed in vacuo in sealed tubes with constant-boiling HCl containing norleucine (5 nmol) as internal standard and 0.01% 2-mercaptoethanol. The tubes were heated for 24 h at 115°C. After evaporation of the acid in vacuo, the samples were analysed by using an amino acid analyser (Beckman 121 MB instrument; Beckman, High Wycombe, Bucks., U.K.).

**Modification of the conjugate with γ-glutamyltransferase**

Solutions of the conjugate were incubated in the presence of monolayer cultures of a rat hepatoma-cell line (J1B1 cells) (Manson et al., 1981). These cells are rich in γ-glutamyltransferase (EC 2.3.2.2) activity (1000 times the activity of normal liver epithelial cells (Manson & Green, 1982)) with over 80% of the activity present on the external surface of the cell membrane (Ding et al., 1981). Confluent monolayers of cells were grown in multi-well plastic
plates in Williams E medium (Manson et al., 1981). Medium was removed and replaced with 1 ml of a solution containing approx. 2 μg of the AFB,-GSH conjugate and 22 mM-glycylglycine in phosphate-buffered saline (0.15 M-NaCl/12 mM-sodium phosphate buffer), pH 6.9, and the cultures incubated at 37°C in O₂/CO₂ (19:1). Portions (250 μl) of the medium were removed at intervals, and samples were prepared and analysed for loss of the conjugate by h.p.l.c. as described above. Controls included incubations in the absence of cells, and in the presence of cells plus 1 mM-compound AT125, a specific inhibitor of γ-glutamyltransferase activity (Reed et al., 1980).

**Results and discussion**

**Formation of the conjugate in vitro**

The system devised for the formation of the conjugate in vitro was capable of the conversion of 80% of the added AFB, to form the conjugate at the concentration of substrate used in the present study during a 30 min incubation period. The overall metabolic conversion can be summarized as follows:

\[
\text{AFB} \downarrow \text{Microsomal fraction} \rightarrow [\text{AFB} \downarrow \text{epoxide}] \rightarrow \text{Cytosol enzyme} \rightarrow \text{AFB} \downarrow \text{GSH}
\]

The system developed in the present study utilized chicken microsomal fraction (3 mg of microsomal protein/assay) for the primary activation step and the mouse cytosol (6 mg of protein/assay) for the conjugative stage. The choice of chicken liver microsomal fraction (in preference to liver microsomal fraction prepared from control or phenobarbitone-prestimulated rats or mice) was based on our previous observations that the chicken microsomal fraction not only metabolized AFB, efficiently under the incubation conditions used (>1.5 nmol of AFB,/min per mg of microsomal protein) but also that in this fraction epoxidation accounted for most of the AFB, metabolized (Neal et al., 1981b). The latter was deduced from the amounts of AFB, 8,9-dihydrodiol formed in incubations, which could be extracted as a complex with Tris buffer (Neal & Colley, 1979a). This compound accounted for over 95% of all the soluble AFB, metabolites produced. AFB, epoxide itself cannot be assayed, as it appears to be extremely labile and rapidly undergoes macromolecular binding or is converted by hydration into AFB, 8,9-dihydrodiol. Mouse hepatic cytosol was found to be the most efficient of those fractions examined (mouse, phenobarbitone-prestimulated rat, chicken) in the conjugative step. The results obtained showed that, on a fresh-tissue-equivalent basis, the mouse cytosol compared with chicken cytosol was far more efficient in conjugating the activated AFB, formed by chicken microsomal fraction in vitro. Cytosol from the liver of phenobarbitone-stimulated rats possessed an intermediate activity between those of the mouse and chicken cytosols.

**Chromatographic properties**

The AFB,-GSH conjugate was subjected to h.p.l.c. with the system described in the Materials and methods section. The conjugate, present in initial samples of incubation media before fractionation of the individual aflatoxin metabolites, exhibits an interesting property that can easily be exploited in the purification of the conjugate. The retention time of the conjugate is approximately doubled by performing the h.p.l.c. at pH 4.0 instead of pH 7.0. If mixtures of metabolites are extracted with chloroform at pH 7.0, and subsequently subjected to h.p.l.c. at pH 4.0, the AFB,-GSH conjugate has a retention time intermediate between those of the non-polar AFB, and of the AFB, metabolites that are removed by the chloroform extraction. The h.p.l.c. of other aflatoxin metabolites and parent aflatoxins is not altered by the acidification. On t.l.c. analysis in a polar solvent system (butanol/acetic acid/water, 2:1:1, by vol.) the purified conjugate gave a single fluorescent spot, which was also ninhydrin-positive (R f 0.28).

**U.v.-spectral properties**

Purified AFB,-GSH conjugate in water exhibited a typical aflatoxin type of spectrum with \( \lambda_{\text{max}} \) at 365 nm. Acidification of the mixture increased the absorbance, and the addition of alkali (pH 10) reversed this such that the \( \lambda_{\text{max}} \) of the alkaline sample was approximately half that of the acidified sample. However, the \( \lambda_{\text{max}} \) was not affected by pH change, and in both acid and alkali conditions maximum absorbance occurred at 365 nm. This contrasts with the u.v.-spectral properties of AFB, 8,9-dihydrodiol, which shows a marked bathochromic shift to 404 nm in basic conditions; this strongly suggests the absence of the hydroxy group in the 8-position (Büchi et al., 1967).

Experiments were performed in which \(^{14}\text{C} \) AFB, was metabolized and the resulting labelled conjugate was purified and subjected to h.p.l.c. A comparison of the absorbance at 365 nm on h.p.l.c. of the conjugate at pH 4.0 and radioactivity content with those of \(^{14}\text{C} \) AFB, chromatographed under similar conditions indicated an absorption coefficient of 19 500–21 500 \text{cm}^{-1} \text{mg}^{-1} \text{cm}^{-1} \text{for the conjugate.}

\(^{1}H-n.m.r.\) and mass spectrometry

Evidence for the structure of the isolated product as an AFB,-GSH conjugate is obtained from its
field-desorption mass spectrum, which shows an abundant MH\(^+\) ion (m/e = 636) associated with a smaller molecular ion at m/e 658 due to Na\(^+\) attachment, MNa\(^+\) (Fig. 1). Further, the fragment ions at m/e = 308 (GSH), 130 and 84 are characteristic of field-desorption mass spectra of GSH conjugates (Przybylski, 1982).

The most intensive ion at m/e = 228 was not definitely assigned and is probably a fragment of the AFB\(_1\) skeleton. This fragment has been observed in the mass spectrum of AFB\(_1\) (Lee et al., 1974). The molecular ion obtained is consistent with a GSH conjugate of AFB\(_1\) formed via an AFB\(_1\) epoxide.

Interpretation of the 360 MHz \(^1\)H-n.m.r. spectrum (Fig. 2) of the purified material was assisted by homonuclear decoupling experiments and by comparison with the 100MHz n.m.r. spectra of the parent compounds (Table 1).

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**Fig. 1. Mass spectrum of AFB\(_1\)–GSH conjugate**

For experimental details see the text.

**Fig. 2. \(^1\)H-n.m.r. spectrum of AFB\(_1\)–GSH conjugate and proposed structure of conjugate**

For experimental details see the text.
The doublet of triplets and triplet at δ 2.06 and 2.44 were assigned to the β- and γ-protons of the glutamyl moiety of the conjugate. The α-protons of glutamic acid and glycine (H\textsubscript{a}), which give a triplet at δ 3.75 (J = 6 Hz) and two-proton singlet at δ 3.91 respectively, in GSH appear to give an overlapping three-proton signal at δ 3.68 in the proposed conjugate. The slight upfield shift of these signals has also been observed in the \textsuperscript{1}H-n.m.r. spectrum of N-(glutathion-S-yl)-2-aminofluorene (Mulder et al., 1982). Comparison with GSH identified the α-proton of cysteine as a triplet at δ 4.50 (J = 6.1 Hz). Irradiation at this position led to the collapse of the two single-proton doublets of doublets at δ 2.79 and 3.09 to two doublets, confirming the assignment of these signals to the β-protons of cysteine. The large difference in chemical shift between these two signals compared with GSH is in agreement with linkage of the glutathione moiety via the sulphur atom. This phenomenon has been observed in other sulphur-linked glutathione conjugates (Mulder et al., 1982; Clinkie et al., 1979).

The strongest signal in the conjugate spectrum is a three-proton singlet at δ 3.93, identified as the aromatic O-methyl resonance of the aflatoxin moiety. A comparison of the conjugate spectrum with that of AFB\textsubscript{1} shows the absence of olefinic coupling in the conjugate, confirming addition at the 8,9-double bond. One-proton singlets at δ 4.59 and 5.46 are identified as the resulting aliphatic protons after addition. These chemical-shift values are consistent with substitution adjacent to the two protons, as demonstrated by the \textsuperscript{1}H-n.m.r. spectra of AFB\textsubscript{1}, 8,9-dihydropyridol (Coles et al., 1980). The total absence of coupling suggests the trans-configuration. Two doublets at δ 4.08 (J = 5.4 Hz) and 6.52 (J = 5.4 Hz) were assigned to the cis-protons (\textit{H}\textsubscript{a} and \textit{H}\textsubscript{m}) of the two furan ring system, and the assignment was confirmed by decoupling. A proton singlet at δ 6.52 was identified as the aromatic proton (\textit{H}\textsubscript{r}).

The aliphatic protons of the cyclopentenone ring were identified by comparison with the spectrum of the parent AFB\textsubscript{1} taken in chloroform, and by comparison with literature values of AFB\textsubscript{1} metabolites obtained in \textsuperscript{2}H\textsubscript{2}O. According to an interpretation of the spectra of the biochemically produced AFB\textsubscript{1}–guanine adduct and the chemically synthesized 8,9-dihydro-9-hydroxy-8-(4-nitrobenzoxo)-AFB\textsubscript{1} (Essigman et al., 1977), the protons at the 2-position and 3-position appeared as multiplets at δ 2.57 and 2.58 and δ 3.22–3.30 respectively. From this information the assignments for the AFB\textsubscript{1}–GSH protons were δ 2.58 (2-position), which appears as a two-proton multiplet, and δ 3.29 (3-position), which appears as a single-proton doublet (J = 6 Hz). The latter observation is con-

### Table 1. \textsuperscript{1}H-n.m.r. data for proposed AFB\textsubscript{1}–GSH conjugate, GSH and AFB\textsubscript{1}

The \textsuperscript{1}H-n.m.r. data for the AFB\textsubscript{1}–GSH conjugate refer to the 360MHz spectrum in \textsuperscript{2}H\textsubscript{2}O. Chemical shifts are given in p.p.m. from an external standard, sodium 3-trimethylsilyl[2,2,3,3-\textsuperscript{2}H\textsubscript{4}]propionate. The \textsuperscript{1}H-n.m.r. data for GSH refer to the 100MHz spectrum in \textsuperscript{3}H\textsubscript{2}O. Chemical shifts are given in p.p.m. from an external standard, sodium 3-trimethylsilyl[2,2,3,3-\textsuperscript{2}H\textsubscript{4}]propionate. The \textsuperscript{1}H-n.m.r. data for AFB\textsubscript{1} refer to the 100MHz spectrum in \textsuperscript{3}H\textsubscript{2}O. Chemical shifts are given in p.p.m. from an external standard, tetramethylsilane. For experimental details see the text.

<table>
<thead>
<tr>
<th>Proton</th>
<th>AFB\textsubscript{1}–GSH conjugate (δ, ppm)</th>
<th>GSH (δ, ppm)</th>
<th>AFB\textsubscript{1} (δ, ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2.06 (2H, d of t, J = 6.3 Hz)</td>
<td>2.09 (2H, d of t, J = 7.3 Hz)</td>
<td>—</td>
</tr>
<tr>
<td>b</td>
<td>2.44 (2H, t, J = 6.3 Hz)</td>
<td>2.45 (2H, t, J = 7.4 Hz)</td>
<td>—</td>
</tr>
<tr>
<td>c</td>
<td>2.58 (2H, m)</td>
<td>—</td>
<td>2.69 (2H, m)</td>
</tr>
<tr>
<td>d\textsubscript{1}</td>
<td>2.79 (1H, J\textsubscript{gem} = 13.5 Hz, J\textsubscript{ag} = 8.4 Hz)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d\textsubscript{2}</td>
<td>3.09 (1H, d of d, J\textsubscript{gem} = 13.5 Hz, J\textsubscript{ag} = 5.1 Hz)</td>
<td>2.88 (2H, d, J = 6.4 Hz)</td>
<td>—</td>
</tr>
<tr>
<td>e</td>
<td>3.29 (1H, d, J = 6 Hz)</td>
<td>—</td>
<td>3.34 (2H, m)</td>
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<tr>
<td>f</td>
<td>3.68 (3H, m)</td>
<td>3.75 (1H, t, J = 6 Hz)</td>
<td>—</td>
</tr>
<tr>
<td>g</td>
<td>3.93 (3H, s)</td>
<td>3.91 (2H, s)</td>
<td>3.97 (3H, s)</td>
</tr>
<tr>
<td>h</td>
<td>4.08 (1H, d, J = 5.4 Hz)</td>
<td>—</td>
<td>4.76 (1H, t of d, J = 2 Hz, J\textsubscript{d} = 6 Hz)</td>
</tr>
<tr>
<td>i</td>
<td>4.52 (1H, t, J = 6 Hz)</td>
<td>4.50 (1H, t, J = 6.1 Hz)</td>
<td>—</td>
</tr>
<tr>
<td>j</td>
<td>4.59 (1H, s)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>k</td>
<td>5.46 (1H, s)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>l</td>
<td>6.52 (1H, s)</td>
<td>—</td>
<td>6.42 (1H, s)</td>
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<tr>
<td>m</td>
<td>6.70 (1H, d, J = 5.4 Hz)</td>
<td>—</td>
<td>6.80 (1H, d, J = 7.3 Hz)</td>
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<tr>
<td>n</td>
<td>—</td>
<td>—</td>
<td>5.47 (1H, t, J = 2.4 Hz)</td>
</tr>
<tr>
<td>o</td>
<td>—</td>
<td>—</td>
<td>6.49 (1H, t, J = 2 Hz)</td>
</tr>
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</table>
sistent with the loss of a proton at the 3-position, and this is confirmed by the absence of geminal coupling.

The absence of an expected proton must raise the possibility that GSH is linked via the 3-position. From the n.m.r. data alone, however, this would appear to be unlikely. Substitution at the 3-position, particularly by sulphur, would have a significant deshielding effect, with resulting downfield shift for the remaining proton (Shoolery, 1959); this was not observed. Further evidence against substitution at C-3 arises from mass-spectral data, i.e. $\text{MH}^+$ ($m/e = 636$). The only C-3-substituted isomer compatible with this molecular weight would involve the addition of water to the 8,9-double bond. The methylene protons arising from such an addition are not observed in the $^1$H-n.m.r. spectrum. The most probable explanation for the loss of a proton at C-3 is that deuterium exchange has occurred by intramolecular base-catalysed keto–enol tautomerism. A molecular model of the AFB$_1$–GSH structure, shown in Fig. 2, confirmed that the free amino group of glutamic acid is spatially capable of lying adjacent to the C-3 position and may catalyse the loss of the proton via deuterium exchange. Attempts to avoid deuterium exchange by using either deuterated acetone or chloroform as solvent were unsuccessful, owing to the low solubility of the conjugate in these solvents. In deuterated dimethyl sulphoxide the protons of interest were hidden by the residual protons of water in the dimethyl sulphoxide.

Addition of GSH at the 8- or 9-position of AFB$_1$ is therefore supported by $^1$H-n.m.r. and mass-spectral data. Hydroxylation at the alternative position is expected from mass-spectral evidence and the proposed mechanisms of formation. The absence of bathochromic shift in the u.v. spectrum under basic conditions suggests that hydroxylation is not at the 8-position. This conclusion would not be inconsistent with the n.m.r. data for the H$_2$ and H$_4$ protons (Fig. 2). Therefore the structure of the GSH conjugate of AFB$_1$ isolated is deduced to be 8,9-dihydro-8-($S$-glutathionyl)-9-hydroxy-aflatoxin B$_1$ (Fig. 2).

Amino acid analysis

The results of amino acid analyses of acid hydrolysates of the conjugate are given in Table 2. These are consistent with the conjugate containing equimolar ratios of glycine and glutamic acid.

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![Image of Fig. 3](image-url)

**Fig. 3. Metabolism of AFB$_1$–GSH conjugate in the presence of $\gamma$-glutamyltransferase activity**

(a) AFB$_1$–GSH conjugate + JB1 cells; (b) AFB$_1$–GSH conjugate + JB1 cells + compound AT125. Samples taken after 180 min were subjected to h.p.l.c. For experimental details see the text. The arrow (●) indicates the position of the AFB$_1$–GSH conjugate. Incubations in the absence of cells gave similar results to (b). Samples taken during initial 120 min of incubation in the absence of compound AT125 exhibited progressive loss of AFB$_1$–GSH conjugate accompanied by appearance of the peak at retention time 12 min.
Table 2. Amino acid analysis of AFB₁-GSH conjugate
For experimental details see the text.

<table>
<thead>
<tr>
<th>Glutamic acid (nmol)</th>
<th>Glycine (nmol)</th>
<th>Glu/Gly molar ratio</th>
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<tbody>
<tr>
<td>1</td>
<td>3.91</td>
<td>4.29</td>
</tr>
<tr>
<td>2</td>
<td>3.86</td>
<td>4.63</td>
</tr>
</tbody>
</table>

γ-Glutamyltransferase experiments

The results of the experiments in which the conjugate was incubated in the presence of high activities of γ-glutamyltransferase (present on the surface of hepatoma cells) show that the conjugate was converted into a compound having a different retention time on h.p.l.c., and that this conversion was not only dependent on the presence of the cells, but also could be blocked by compound AT125, the specific inhibitor of γ-glutamyltransferase activity (Fig. 3). These data are consistent with the proposed structure, in which the conjugate possesses a potentially transferable γ-glutamyl moiety.

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References