Introduction

The alteration of cellular metabolic processes are very important indices in the aetiology of chronic degenerative disease processes in man and animal systems (Boyd et al., 1982) In both carcinogenic and non-carcinogenic disorders of the liver, the underlying biochemical lesion is the derangement (involving either inhibition or activation) of one or more essential metabolic processes of organelles in the hepatocyte. Mitochondrial damage is believed to be one of the primary events in the genesis of hepatocyte damage (Kulkarni and Hudgson, 1980). Such damages could be elicited by a number of xenobiotics (such as the aflatoxins) present in foods (WHO 1979).

The coumarin compounds aflatoxin B₁ (AFB₁) and scopoletin are known food contaminants (Sargeant et al., 1961; Minamikawa et al., 1967). They could be retained in human body when taken through diet (Hendrickse et al., 1983; Obasi and Obidoa, 1995).

In particular, traces of AFB₁ have been detected in the liver and muscle samples of cows slaughtered in our locality (unpublished laboratory data). Both AFB₁ and scopoletin could be ingested by the bovine species through diet (Uzoho, 1991) hence the curiosity as to the possible effects of both compounds in the bovine species.

In our previous studies, we showed that both AFB₁ and scopoletin interfered with bovine hepatic mitochondrial respiration in vitro (Obasi and Obidoa, 1997a). Interference with mitochondrial respiration could imply that the compounds interacted with a number of the enzymatic processes of respiration, particularly the respiratory complexes. Earlier, we have described the effects of both scopoletin and AFB₁ on cow liver mitochondrial  α-ketoglutarate dehydrogenase (complex I) and succinate dehydrogenase (Complex II) (Obasi and Obidoa, 1994). We are progressing with the systematic investigations of the in vitro effects of these two compounds on the components of the bovine hepatic mitochondrial respiratory chain, with a view to understand the pattern of the effects of the compounds in an animal like the bovine species. In the present study, we have compared the effects of AFB₁ and scopoletin on the bovine hepatic mitochondrial respiratory complex III enzymes, the a-ketoglutarate cytochrome c and succinate cytochrome c reductases.

Materials and Methods

Chemical and reagents

The common reagents and chemicals used in these experiments were of analytical grade, the
highest purity available, and obtained from Sigma Co (St. Louis, USA), BDH (Poole, England) or Merck (Darmstadt, Germany).

The purity of both scopoletin (Serva Fein-Biochemica, Heidelberg) and AFB$_1$ (Sigma) was in each case, determined by the production of a single bluish fluorescent (UV-short wavelength) spot when the compound was subjected to thin-layer chromatography using methanol in chloroform (3:97 v/v) as the development solvent (Obidoa and Obasi, 1991).

Isolation of bovine hepatic mitochondria

Liver samples (approximately 20 g) were freshly obtained from male cow (Bos indicus) slaughtered at the local abattoir. The liver samples (collected within 10 min after decapitating the animals) were placed in ice-cold 0.34 M sucrose. After peeling off the outer coat of the liver (liver pulp), weighed fractions of the samples were minced and washed with 0.34 M sucrose solution. Mitochondria were subsequently isolated from washed liver slices following the procedures described by Johnson and Lardy (1967) using 0.34 M sucrose as isolation medium. Isolated mitochondria were re-suspended in 0.34 M sucrose – 0.02 M Tris (hydroxy-methyl) aminomethane-HCl buffer (pH 7.4). Mitochondrial protein was determined by the Biuret method (Thorne, 1984).

Measurement of the enzyme activities

The liver mitochondrial cytochrome $c$ reductase activities were assayed spectrophotometrically using ferricytochrome $c$ as the terminal electron acceptor (oxidant) by a modified method of Obidoa et al. (1978). The reaction medium (final volume of 3.0 ml) consisted of 100 mM potassium phosphate buffer pH 7.4, 1.33 μM ferricytochrome $c$; and 2.5 mg mitochondrial protein preincubated for 2 min in freshly neutralized 20 mM potassium cyanide to inhibit the cytochrome oxidase. The concentrations indicated were the final concentrations in the reaction medium. The effects of the toxins on the enzyme activities were determined by adding the appropriate amounts of the toxins to the reaction medium, while 10% N,N$^1$-dimethyl formamide (DMF) solution of equivalent volume was added in control experiments. The reaction was initiated by adding either succinate (final concentration in reaction medium was 20 mM) for succinate-cytochrome $c$ reductase, or $a$-ketoglutarate (final concentration in reaction medium was also 20 mM) for $a$-ketoglutarate cytochrome $c$ reductase, to the reaction medium. Oxidized NAD$^+$ (final concentration in reaction medium was 10 mM) was added as a coenzyme prior to the addition of $a$-ketoglutarate in the $a$-ketoglutarate cytochrome $c$ reductase assay. Absorbance changes of the assay medium were recorded at 550 nm for 6 min in a Milton Roy Spectronic 1201 UV-visible split beam spectrophotometer set at the kinetic mode. Specific activities of the enzyme (v) were calculated from the following relationship (Obi, 1985).

$$v = \frac{OD/t}{P E}$$

where

$P$ = protein concentration,

$E$ = ferricytochrome $c$ extinction coefficient (18.5 $m^{-1} cm^{-1}$)

$OD/t$ = absorbance change per unit time.

Enzyme activity (v) was expressed as nmol Fe$^{3+}$ cytochrome $c$ reduced per minute per mg mitochondrial protein (mmp). i.e., nmol Fe$^{3+}$ Cyt $c$ reduced $\times$ min$^{-1}$ $\times$ mmp$^{-1}$.

Preincubation experiments were carried out by first incubating the appropriate amount of the coumarin (scopoletin or AFB$_1$) compound to be tested (or equivalent volume of aqueous 10% DMF solution for control), with the needed amount of mitochondrial suspension for 2–3 min before the other components of the reaction medium were added.

All other assay procedures were as described earlier.

Analysis

All statistical analysis were done by Students “t” test.

Results and Discussion

Effects of Scopoletin and AFB$_1$ on Bovine Hepatic Mitochondrial $a$-ketoglutarate cytochrome $c$ reductase

Both scopoletin and AFB$_1$ stimulated bovine hepatic mitochondrial $a$-ketoglutarate cytochrome $c$ reductase at the toxin concentrations (5–20 nmol/mmp) studied.
However, the stimulatory effects of scopoletin (8–33%) were higher than those of AFB₁ (5–23%) at equivalent concentration (Table I). Apart from the effects of the 10 nmol/mmp concentration of the toxins, the difference between the effects of scopoletin and AFB₁ on α-ketoglutarate-cytochrome c reductase were not significant (p>0.05). However, the stimulatory effects of both scopoletin and AFB₁ on the enzyme were significant (p<0.05) relative to the control from 10–20 nmol/mmp concentrations.

Changes of the kinetic parameters (\(K_m\) and \(V_{max}\)) calculated by direct liner plots according to Eienthal and Cornish-Bowden (1974) are shown in Table II. The results show that in the presence of the toxins, the \(K_m\) values generally decrease relative to the control (p<0.05), while the \(V_{max}\) slightly increased (p>0.05).

These effects were concentration-dependent, and more pronounced with scopoletin than with AFB₁. This is in agreement with the observed stimulatory effects of the toxins.

However, the values for the lower AFB₁ concentrations (5–10 nmol/mmp) indicated decreases relative to the control values.

On pre-incubating the toxins with mitochondria and assaying for the enzyme activity, an enhancement of the stimulatory effects of both AFB₁ and scopoletin relative to the non-preincubated condition was observed. The increases in the enzyme activities due to pre-incubation were generally significant (p<0.05) relative to the corresponding non-preincubated values. The enhancement was more for AFB₁ (20–39% increase) than for scopoletin (5–23% increase).

### Effects of scopoletin and AFB₁ on bovine hepatic mitochondrial succinate cytochrome c reductase

The bovine hepatic mitochondrial succinate-cytochrome c reductase was generally inhibited by both scopoletin and AFB₁. However, the inhibitory effects of scopoletin on the enzyme were found to be inversely proportional to the toxin concentration. On the other hand, the inhibitory effects of AFB₁ on the enzyme was directly proportional to the toxin concentration although the lower (5.0 nmol/mmp) concentration of the toxin exhibited small stimulatory effects on the enzyme (Table III).

At equivalent concentrations (10–20 nmol/mmp), AFB₁ exhibited higher inhibitory effects (10–20%) on the succinate-cytochrome c reductase than scopoletin (1–8%).

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### Table I. % Change in the mean activities of bovine hepatic mitochondrial α-ketoglutarate-cytochrome c reductase in the presence of scopoletin and aflatoxin B₁ (AFB₁).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Non-preincubated</th>
<th>Preincubated</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 nmol scopoletin per mmp</td>
<td>+8.0</td>
<td>+13.9</td>
</tr>
<tr>
<td>10 nmol scopoletin per mmp</td>
<td>+20.1</td>
<td>+33.8</td>
</tr>
<tr>
<td>20 nmol scopoletin per mmp</td>
<td>+33.2</td>
<td>+50.6</td>
</tr>
<tr>
<td>5 nmol AFB₁/mmp</td>
<td>+5.0</td>
<td>+28.0</td>
</tr>
<tr>
<td>10 nmol AFB₁/mmp</td>
<td>+10.4</td>
<td>+49.0</td>
</tr>
<tr>
<td>20 nmol AFB₁/mmp</td>
<td>+22.9</td>
<td>+58.8</td>
</tr>
</tbody>
</table>

The mean activities (velocity) are expressed as nmole Fe³⁺Cyt reduced × min⁻¹ × mmp⁻¹. The values were calculated in the presence of different concentrations (5–20 nmol/mmp of scopoletin and AFB₁, at fixed substrate concentration [S] of 1.33 μM. Enzyme activities are expressed as mean ± S.D (n=6). % change in activities due to the coumarins were calculated from the expressions \(B - A \times 100\) where A and B are the mean enzyme activities in the absence and presence of the coumarins, respectively. + sign indicates stimulation.

### Table II. Summary of the effects of scopoletin and aflatoxin B₁ on bovine hepatic mitochondrial α-ketoglutarate cytochrome c reductase.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>(K_m) [μM]</th>
<th>(V_{max}) (nmol Fe³⁺Cyt reduced × min⁻¹ × mmp⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10% DMF)</td>
<td>2.10±0.95</td>
<td>5.55±1.02</td>
</tr>
<tr>
<td>5 nmol scopoletin</td>
<td>0.60±0.05</td>
<td>5.50±1.21</td>
</tr>
<tr>
<td>10 nmol scopoletin</td>
<td>0.90±0.14</td>
<td>6.15±0.93</td>
</tr>
<tr>
<td>20 nmol scopoletin</td>
<td>0.59±0.02</td>
<td>6.00±1.10</td>
</tr>
<tr>
<td>5 nmol AFB₁</td>
<td>1.40±0.86</td>
<td>5.10±1.14</td>
</tr>
<tr>
<td>10 nmol AFB₁</td>
<td>1.25±0.42</td>
<td>5.80±1.28</td>
</tr>
<tr>
<td>20 nmol AFB₁</td>
<td>1.10±0.16</td>
<td>6.00±0.25</td>
</tr>
</tbody>
</table>

The values represent mean ± S. D. (n=6).
Table III. % Change in the mean activities of bovine hepatic mitochondrial succinate-cytochrome c reductase in the presence of scopoletin and aflatoxin B1.

<table>
<thead>
<tr>
<th>Concentrations of the compounds [nmol/mmp]</th>
<th>Km [μM]</th>
<th>Vmax (nmol Fe3⁺ x min⁻¹ x mmp⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10% DMF)</td>
<td>1.40 ±0.82</td>
<td>6.25 ±1.20</td>
</tr>
<tr>
<td>5 nmol Scopoletin</td>
<td>1.80 ±0.56</td>
<td>5.80 ±1.61</td>
</tr>
<tr>
<td>10 nmol Scopoletin</td>
<td>2.40 ±0.25</td>
<td>6.40 ±1.72</td>
</tr>
<tr>
<td>20 nmol Scopoletin</td>
<td>1.60 ±0.34</td>
<td>6.50 ±0.95</td>
</tr>
<tr>
<td>5 nmol AFB1</td>
<td>2.20 ±0.45</td>
<td>7.0 ±1.95</td>
</tr>
<tr>
<td>10 nmol AFB1</td>
<td>1.50 ±0.32</td>
<td>5.65 ±0.98</td>
</tr>
<tr>
<td>20 nmol AFB1</td>
<td>3.1 ±0.76</td>
<td>5.90 ±0.75</td>
</tr>
</tbody>
</table>

The values represent mean ± S.D. (n=6).

These results showed that by using kinetic studies, it could be established that both scopoletin and AFB1 stimulated bovine hepatic mitochondrial succinate-cytochrome c reductase activities in vitro. The increase in the stimulatory effects of the toxins on a-ketoglutarate cytochrome c reductase, and the reversal of their inhibitory effects on succinate cytochrome c reductase by prolonged exposure of the mitochondria to the toxins (pre-incubation) emphasizes the important roles which the membrane plays on the effects of these toxins, since succinate dehydrogenase is membrane bound and a-ketoglutarate dehydrogenase is located in the matrix. These effects could result from the symmetric orientation, restriction, and plasticity of membrane bound enzymes as indicated by Obidoa et al. (1980). Consequently, the penetration of the mitochondrial membrane by these toxins as had been suggested (Obasi and Obidoa, 1997b) could cause alterations in the molecular architecture or orientation and function of the membrane bound proteins such as succinate dehydrogenase enzyme proteins. These observations could however, be confirmed by the assay of the purified enzymes alone and in reconstituted systems.

When taken with our previous reports on the effects of these toxins on the bovine hepatic mitochondrial respiration, and complex I enzymes (Obasi and Obidoa, 1995, 1997a), these results further indicate that at very minute levels, scopoletin and AFB1 could elicit alterations in the bovine hepatic mitochondrial respiratory activities.

The mean activities (velocity) are expressed as nmol Fe³⁺ Cyt.c red x min⁻¹ x mmp⁻¹. The values were calculated in the presence of different concentrations (5–20 nmol/mmp) of scopoletin and AFB1, at a fixed substrate concentration [S] of 1.33 mM. Enzyme activities are expressed as mean ± S.D. (N=6). % change in activity were calculated as indicated in Table I legend. + Sign indicates stimulation; while – sign indicates inhibition.

Although these inhibitions were generally not significant (P>0.05) except apparently, for that of 20 nmol/mmp AFB1, the inhibitory effects of AFB1 on the enzyme were generally significantly (P<0.05) higher than those of scopoletin at equivalent concentrations of the toxins.

Changes in the kinetic parameters (Km and Vmax) as shown in Table IV, indicate a general increase in the Km values of the enzyme in the presence of the toxins. The calculated Vmax from these plots, however, indicated values consistent with the observed inhibitory/stimulatory (for 5 nmol/mmp AFB1) effects of AFB1 on the enzyme. On the other hand, the calculated Vmax values for higher scopoletin concentrations (10–20 nmol/mmp) deviated from the observed inhibitory effects. Likewise, the calculated Km values in the presence of 5 nmol/mmp AFB1 was not consistent with the observed stimulatory effects (higher Vmax values).

Pre-incubating the mitochondria with the toxins generally reversed the inhibitory/stimulatory effects of the various concentrations of both toxins (Table III).


