SECTION I

TRANSFORMATION OF BENZO(a)PYRENE (BP) BY ASPERGILLUS OCHRACEUS TS IN VIVO

Characterization of BP Metabolites
To make a probe about metabolism of benzo(a)pyrene by *Aspergillus ochraceus* TS transformation was tried in liquid culture under *in vivo* conditions. Again, in order to understand the pathway of polycyclic hydrocarbon degradation particularly the routes leading to detoxification as well as carcinogen activation attempts have been made to rapidly isolate and characterize the benzo(a)pyrene metabolites by various physico-chemical means.
MATERIALS AND METHODS

Vegetative cell fermentation

The test organism, Aspergillus ochraceus TS was cultivated in 100 ml of sterile (sterilized at 15 p.s.i. for 15 min.) medium (composition, % w/v : sucrose 1; cornsteep liquor, 0.5; K$_2$HPO$_4$, 0.05; pH 6.5 before sterilization) taken in Erlenmeyer flask (500 ml). The sterile medium was inoculated with spore suspension obtained from week-old cultures maintained on agar slant of the same medium. The volume of inoculum (1 ml) containing 8 x 10$^8$ cells was used in all experiments unless otherwise stated. The organism was allowed to grow at 28-30°C on a rotary shaker for 48 h. The mycelium was then filtered and transferred to fresh medium. This was followed by the addition of BP dissolved in dimethyl formamide (DMF) (0.4 mM, 10 mg) for further incubation for 48 h. Control experiments were run in parallel both in the absence of the organism and substrate. All the flasks were covered with black paper during fermentation.

Isolation of BP metabolites

After the fermentation the mycelium was filtered, washed successively with 0.5% (w/v) NaCl solution and water to make them free from adhering substrate. The washings pooled in the
filtrate were then extracted with half the volume of ethyl acetate three times. The organic extract was washed with water, dried over anhydrous Na₂SO₄ and finally evaporated to dryness under reduced pressure and temperature (yield 45%).

Characterization of BP metabolites

The crude residue dissolved in methanol was resolved on silica gel G plates (TLC) using benzene : methanol (95 : 5) as solvent system. The chromatogram was developed in an iodine chamber. The synthetic BP derivatives were used as reference.

High pressure liquid chromatography (HPLC) of BP metabolites

High pressure liquid chromatography (HPLC) was used for resolution and isolation of BP-metabolites. This was done with a Waters model 440 chromatograph fitted with a Bondapak C₁₈ analytical column (reverse phase, 0.7 x 25 cm) in conjunction with a programmed solvent gradient. The initial and final solvent compositions were methanol : water (50 : 50) and (90 : 10) respectively. A convex gradient (curvature setting 5) was employed with a flow rate of 2 ml/min at 3000 p.s.i. The column temperature was maintained at 25°C. The eluate was monitored at 280 nm with a uv detector.
The retention time of BP metabolites were determined using synthetic BP derivatives as reference and thus their identity was established.

**Acid catalysed dehydration of 9,10-dihydrodiol formed from BP by A. ochraceus TS**

BP-metabolite (9,10-dihydrodiol) was dissolved in methanol to which 0.05 ml of 3 N HCl was added. Phenol formation from the dihydrodiol was followed by measuring the increase in absorbance at 375 nm. Synthetic trans-BP-9,10-dihydrodiol was treated similarly.

In another experiment, the methanolic solution of 9,10-dihydrodiol formed from BP by A. ochraceus TS was treated with 0.1 ml of 6 N HCl and heated at 80°C for 15 min., then the spectrum was recorded in situ in a Beckman spectrophotometer.

Further characterization of BP-metabolites was achieved with the help of their absorbance (UV) and fluorescence spectra. While the absorption spectra was recorded in a Beckman spectrophotometer, the fluorescence spectra was measured on a Perkin-Elmer MPF 44 B fluorescence spectrophotometer. For comparison with authentic BP-derivatives they were dissolved in a mixture
of n-hexane : acetone (2:1) which was then extracted with NaOH (1 N). The fluorescence was measured with the alkali extract of the individual authentic sample.

RESULTS

The crude residue showed five bands on silica gel G plate (TLC) including the unreacted substrate (BP). These bands corresponded to the respective synthetic BP-quinones, phenols and dihydrodiols used as reference (Fig. 1.1). A major band below dihydrodiol region was tentatively labeled as the pre-diol region.

The individual band on TLC plate was scraped and extracted with methanol. The methanol extract from several chromatograms were pooled in order to obtain good amount of material for analysis by high pressure liquid chromatography. Resolution of the diol region revealed the presence of three components which were trans-BP-9,10-dihydrodiol, trans-BP-4,5-dihydrodiol and trans-BP-7,8-dihydrodiol (Fig. 1.1a) (Table 1.1). The trans-configuration was established by comparing the relative rates of dehydration of the fungal metabolite with synthetic trans-dihydrodiol(Fig. 1.2). The dehydration experiment was done with 9,10-dihydrodiol since
this metabolite was obtained in appreciable amount only. The metabolite, BP-9,10-dihydrodiol had a similar rate of dehydration to that observed for synthetic trans-BP-9,10-dihydrodiol. Moreover, 9,10-dihydrodiol formed from BP by A. ochraceus TS on treatment with acid furnished a product whose absorption spectrum was identical with that of 9-hydroxy benzo(a)pyrene (Fig. 1.2a). This was further confirmed by high pressure liquid chromatography. The retention times of three fungal dihydrodiols were exactly identical with that of respective synthetic-trans-dihydrodiols.

Analysis of the quinone band of the thin layer chromatogram (Fig. 1.1) by high pressure liquid chromatography showed the presence of two peaks identical with synthetic 1,6 and 3,6-quinones (Fig 1.1b). Again, resolution of the phenol region by HPLC also disclosed the presence of two peaks identical with synthetic 3-hydroxy and 9-hydroxy benzo(a) pyrene (Fig. 1.1c). The retention time of quinones and phenols exactly matched with that of the authentic samples. Figure 1.3 and 1.4 showed the resolution of BP-metabolites as mixture and the synthetic standards by HPLC while Fig. 1.5 represents co-elution profile of BP-metabolite(s) with synthetic standards. The low intensity profile of the
unreacted substrate in the chromatogram (Fig. 1.3) was due to its partial removal from the crude metabolite mixture by treatment with petroleum ether (60-80°C).

In addition to thin layer (TLC) and high pressure liquid chromatography (HPLC) BP metabolites were further characterized by their fluorescence and ultraviolet absorption spectra using synthetic BP-derivatives as reference. Although the fluorescence spectra (Fig. 1.6 and 1.7) established the presence of three types of products in BP metabolites, the ultraviolet absorption profile of the metabolites (Fig. 1.8) were observed to be identical with that of the respective authentic BP derivatives. The products formed from BP by A. ochraceus TS are presented in Figure 1.9. The control experiments in which benzo(a)pyrene was incubated simply in the medium in absence of the organism showed no degradation of the substrate and in absence of the substrate (BP) the organism did not produce anything.
Table 1.1 The retention time of benzo(a)pyrene metabolites separated by high pressure liquid chromatography

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time (min)</th>
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<tbody>
<tr>
<td>9,10-diol-BP</td>
<td>6.26</td>
</tr>
<tr>
<td>4,5-diol-BP</td>
<td>9.9</td>
</tr>
<tr>
<td>7,8-diol-BP</td>
<td>11.0</td>
</tr>
<tr>
<td>1,6-quinone-BP</td>
<td>15.8</td>
</tr>
<tr>
<td>3,6-quinone-BP</td>
<td>16.75</td>
</tr>
<tr>
<td>9-OH-BP</td>
<td>21.25</td>
</tr>
<tr>
<td>3-OH-BP</td>
<td>22.0</td>
</tr>
<tr>
<td>BP</td>
<td>32.0</td>
</tr>
</tbody>
</table>
9.1.1 Thin layer chromatographic analysis of metabolites formed from BP by *A. ochraceus* TS.

1. BP-1,6-Quinone
2. BP-3,6-Quinone
3. 9-Hydroxy BP
4. 3-Hydroxy BP (authentic)
5. BP-Metabolites
6. BP-9,10-Diol
7. BP-7,8-Diol
8. BP-4,5-Diol
9. Benzo(a)pyrene (authentic)
Fig. 1.1 HPLC Elution profile of BP-metabolites a) diol; b) quinone & c) phenol region.
Fig. 1.2 Relative rate of dehydration of dihydrodiol formed from BP by \textit{A. ochraceus} TS; (○-○) Synthetic \textit{trans} BP 9,10-dihydrodiol, (●-●) BP 9,10-dihydrodiol formed by \textit{A. ochraceus} TS.
Fig. 1.2(a) Absorption Spectrum of acid-catalyzed dehydration product of trans
BP-9,10-Dihydrodiol formed from BP by A. ochraceus TS.
Fig. 1.3 HPLC Elution profile of BP metabolites by Aspergillus ochraceus T5.
Fig. 1.4 Resolution of a mixture of synthetic BP derivatives by HPLC.
Fig. 1.5 HPLC Co-elution profile of BP metabolites (-) with synthetic standards (--);
a) diols, b) quinones & c) phenols.
Fig. 16 Excitation Spectra of (a) 7,8-Diol, (b) 1,6-Quinone, (c) 3-OH-BP, (d) BP-Metabolite. (--) Authentic.
Fig. 1.7 Emission Spectra of (a) trans-BP 7,8-dihydrodiol, (b) BP 1,6-quinone, (c) 3-OH BP; (—) BP Metabolite, (—) Synthetic.
Fig. 18a. Ultraviolet spectra of trans-BP 9,10-dihydriodiol (−)
BP Metabolite, (---) Authentic.
Fig. 1.8b. Ultraviolet spectra of trans-BP 7,8-dihydrodiol, (—) BP Metabolite, (---) Authentic.
Fig. 1-8c. Ultraviolet spectra of trans-BP 4,5-dihydrodiol, (---) BP Metabolite, (---) synthetic.
Fig. 1.8d. Absorption spectra of BP 1,6-quinone, (--)
BP Metabolite, (---) synthetic.
Fig. 18e. Absorption spectra of BP 3,6-quinone; (-) BP Metabolite, (--) synthetic.
Fig. 1.8f. Ultraviolet spectra of 3-OH BP, (-- BP Metabolite, (--) Authentic.
Fig. 1* Metabolic profile of BP by Aspergillus ochraceus TS.