1. The present investigation deals with the biochemical properties of aryl hydrocarbon hydroxylase (a cytochrome P-450 monoxygenase) also called BP-hydroxylase produced by *Aspergillus ochraceus* TS, the test organism, capable of metabolizing benzo(a)pyrene under liquid culture conditions. The bio-converted products were isolated by solvent extraction of culture filtrate. The metabolites were resolved on silica gel G plates (TLC) along with authentic standards used as reference. Further, characterization and identity of the metabolites were established with the help of high pressure liquid chromatography (HPLC). HPLC elution profile indicated the presence of three dihydrodiols, two quinones and two phenols including the unreacted substrate, benzo(a)pyrene (BP). The retention time of different diols, quinones and phenols exactly matched with that of the corresponding authentic samples. All the metabolites were completely separated from one another by HPLC contrary to the TLC analysis where they are very often incompletely resolved. The metabolites are
trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene, trans-4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene, trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, BP-1,5-quinone, 3,6-quinone, 3-hydroxy and 9-hydroxy BP. Thus the K-region diol (4,5-diol) is also produced by A. ochraceus TS contrary to the earlier reports on BP metabolism by fungi. However, diols are formed in major amount in comparison to phenols and quinones. The control experiments in absence of organism and in absence of substrate, BP, did not produce anything.

2. The BP-hydroxylase produced by A. ochraceus TS is inducible in character as envisaged by the results of transformation in vitro. There was a seven fold increase in hydroxylase activity in cells pretreated with BP vis-a-vis its control. The enzyme was found to be present in microsomes (105,000 g pellet) which also retained highest titre of NADPH Cyt c reductase. Neither mitochondrial pellet nor post microsomal supernatant (PMS) had any activity. The involvement of Cyt P-450 in this hydroxylation was substantiated by the CO-difference spectrum of both the microsomes and its solubilized preparation from BP induced cells of A. ochraceus TS.

The enzyme has got a definite induction time (16-18 h.) and cells harvested from exponential growth phase had maximum BP-hydroxylase activity.
3. The BP hydroxylation was linear up to 30 min. after which it did not increase significantly. The pH optimum of the BP-hydroxylase was 8.0-8.2. However, a pH of 7.6 was used in all cases for comparison of the data with other systems. The activity at pH 7.6 was 80% of the optimal activity. The optimum temperature was 30°C. Preincubation at 45°C for 15 min. showed 50-60% less activity than at 30°C. Again, the BP-hydroxylase activity in the microsomes was decreased by 25-50% after standing at 4°C for 24 h. while the same stored at -20°C retained 60% of the original activity after 3 to 4 weeks. The BP-hydroxylase of A. ochraceus TS was NADPH dependent and requires molecular oxygen. However, oxidizing agents like NaIO₄ and NaClO₂ could support this hydroxylation indicating the involvement of Cyt P-450 in this hydroxylation. NADH was less effective. Ascorbate and tetrahydrocholate could not serve as cofactors. No synergistic effect was observed when NADH was added along with NADPH. Again, there was no significant increase in BP-hydroxylation by the microsomes prepared from cells grown in presence of different concentration of glucose. The microsomal BP-hydroxylase activity was inhibited to a significant extent by carbon monoxide (CO), metyrapone, SKF-525A, imidazole, PCMB but not by cyanide, azide or antimycin A, the typical
inhibitors of the mitochondrial electron transport chain, indicating the involvement of cytochrome P-450. The inhibitory effect of cytochrome c is consistent with the participation of a Cyt c reductase in this hydroxylation. The stimulatory effect of BSA is possibly due to the increased solubility of BP in aqueous reaction mixture.

Among the metal ions tested, Co\(^{+2}\) and Cu\(^{+2}\) exhibited considerable inhibition. BP-hydroxylation in vitro was NADPH dependent and was found to be activated by G-6-P and G-6-P-dehydrogenase, a NADPH regenerating system. Thus the involvement of G-6-P dehydrogenase in this hydroxylation was indicated. The level of G-6-P-dehydrogenase was increased in both microsomes and PMS of induced cells than the non induced one. Moreover, the kinetic parameters of G-6-P-dehydrogenase in microsomes against G-6-P and NADP as substrates were found to be improved by different inducers.

4. In the present study during the probe on multiple forms of Cyt P-450 catalysing BP-hydroxylation with different classical mammalian Cyt P-450 inducers it was observed that there was a striking resemblance between this system and that obtained with hepatic microsomes and hamster fetus cells. 3-Methylcholanthrene, benzo(a)pyrene, A-naphthoflavone and other aryl
hydrocarbons used as inducers in the range of 80 to 100 μM concentration enhanced the BP-hydroxylase activity. This may be considered to be due to selective induction of a form or forms of Cyt P-450 with a high activity towards BP-hydroxylation. On the other hand, phenobarbital (PB), polychlorinated biphenyl (Aroclor 1254, PCB) and progesterone did induce the BP-hydroxylase to some extent, although a significant induction of NADPH Cyt c reductase activity was observed (3 to 5 fold). Furthermore the inducer added at zero time caused no significant induction but produced a decrease in cell population.

Addition of BP at different concentrations (40-160 μM) during the induction period had a dramatic effect on the kinetics of BP-hydroxylase activity. It was observed that with increase in concentration of BP in the medium there was a lowering of Michaelis constant ($K_m$) i.e. increasing affinity for BP and elevation of maximal velocity ($V_{max}$). Pretreatment with 3-MC, BNF and other aryl hydrocarbons, also resulted a noticeable improvement of $K_m$ and $V_{max}$ of BP-hydroxylase which may be presumed to be due to selective induction of a form (or forms) of Cyt P-450 with a high activity towards BP-hydroxylation. This was substantiated by the fact that these agents do not increase the level of NADPH Cyt c reductase, a key enzyme of the P-450 containing monoxygenase, thus
resembling the general characteristic of inducers of relatively narrow specificity (Cyt P-448). On the other hand, PB, PCB and progesterone induced the BP-hydroxylase to some extent as evidenced by slight modification of $K_m$ and $V_{max}$; although a significant induction of NADPH Cyt c reductase (2.5 to 5 fold) was observed which is believed to be common with inducers of broad specificity (Cyt P-450). Both PB and 3-MC induced microsomes exhibited characteristic metyrapone difference spectra and the results seemed to be consistent with that obtained with purified Cyt P-450 from PB and 3-MC treated rats. Thus the existence of multiple forms of Cyt P-450 in *A. ochraceus* TS metabolising BP was established. Furthermore, flavone was used to probe the relative distribution of different forms of Cyt P-450 in various microsomal preparations obtained from cells induced by different agents. It was observed that both 3-MC and BP-induced microsomal BP-metabolism were inhibited by flavone indicating its similarity with that in rat liver microsomes after 3-MC treatment and also with Cyt P-450 LM$_6$ which is produced in rabbit liver after induction with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). On the other hand, the addition of flavone (0.2-1 mM) to the PB and BNF induced microsomes stimulated the BP metabolism by 2 to 3 fold and thereby exhibited its similarity with Cyt P-450 LM$_4$ which is
the major form produced in rabbit liver after treatment with BNF and TCDD. The results obtained with PCB induced microsomes was really puzzling as the BP metabolism was found to be inhibited at low and activated at high concentration respectively. Presumably, this may be due to the synthesis of different forms of Cyt P-450 to varying extent, since induction by PCB mimicks that observed due to combined administration of PB and 3-MC.

5. Attempts were also made to purify the enzyme system for better understanding of the individual components. Prior to purification attention was paid to develop a good solubilizing technique just because the fungal microsomes containing Cyt P-450 are highly particulate in nature and during solubilization there is every possibility of denaturation of Cyt P-450 to P-420, the inactive form. In the present study, both ionic and non-ionic detergents were tried during solubilization. It was observed that microsomes were effectively solubilized with 0.6% sodium cholate. The cholate solubilized extract was then purified by DEAE-cellulose and hydroxyapatite chromatography. The Cyt P-448 in purified protein had a specific content of 10.2 nmoles/mg protein and the yield was about 40%. The molecular weight of Cytochrome P-448 estimated by SDS-polyacrylamide gel electrophoresis (8%) was found to be \(~ 45,000\).
The purified Cyt P-448 in the oxidised state had absorption maxima at 417 (Soret band), 568 and 534 nm (α,β bands) respectively while its reduced form exhibited the absorption maxima at 412 and 545 nm. The reduced-CO difference spectra of this protein showed absorption maxima at 448 and 550 nm.