Biotransformation of Polycyclic Aromatic Compounds by Fungi and An Investigation into the Oxidation of Alkylbenzenes by *Mortierella isabellina* NRRL 1757

by

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A B S T R A C T

Incubations of several polycyclic heteroaromatic compounds and two polycyclic aromatic hydrocarbons with a series of common fungi have been performed. The fungi Cunninghamella elegans ATCC 26269, Rhizopus arrhizus ATCC 11145, and Mortierella isabellina NRRL 1757 were studied in this regard. Of the aza heteroaromatics, only dibenzopyrrole gave a ring hydroxylated product following the incubation with C. elegans. From the thio heteroaromatics studied, dibenzothiophene was metabolized by all the three fungi and thioxanthone by C. elegans and M. isabellina giving sulfones and sulphoxides. Thiochromanone was metabolized stereoselectively to the corresponding sulphoxide by C. elegans. Methyl substituted thioxanthones on incubation with C. elegans produced oxidative products, arising from S -oxidation and hydroxylation at the methyl group. Of the cyclic ketones studied, only fluorenone was reduced to hydroxyfluorene and this metabolism is compared with that reported with cytochrome P-450 monooxygenases of hepatic microsomes.

A series of para-substituted ethylbenzenes has been transformed stereoselectively to the 1-phenylethanols by incubation with M. isabellina. Comparisons of the enantiomeric purities obtained from products with their respective para substituent of the same steric size but different electronic properties indicate that the stereoselectivity of hydroxylation at benzylic carbon may be susceptible to electron donating or withdrawing factors in some cases, but that observation is

not valid in all the comparisons. The stereochemistry of the reaction is discussed in terms of three possible steps, ethylbenzene ⟶ 1-phenylethanol ⟶ acetophenone ⟶ 1-phenylethanol. This metabolic pathway could account for the inconsistencies observed in the comparisons of optical purities and electronic character of para substituents. Furthermore, formation of 2-phenylethanol (in some cases), 1-(p-acetylphenyl)ethanol from p-diethylbenzene, and N-acetylation of p-ethylaniline was observed. n-Propylbenzene was also converted to optically active 1-phenylpropanol.

Acetophenone, p-ethylacetophenone, and α,α,α-trifluoroacetophenone were transformed to 1-phenylethanol, 1-(p-ethylphenyl)ethanol, and 1-phenyl-2,2,2-trifluoroethanol, respectively, with high chemical and excellent optical yields.

The ^{13}C NMR spectra of several substrates and metabolic products have been reported and assigned for the first time.

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To

My Parents

Who made it all possible

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PART ONE

Biotransformation of Polycyclic Aromatic Compounds

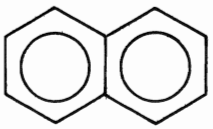
Polycyclic aromatic hydrocarbons (PAHs) are found in air, soils and sediments around the world.[1] They are in great variety (Fig. 1), of unusual chemical stability, and their origins are widespread.

Polycyclic aromatic molecular structures are formed whenever organic substances are exposed to high temperatures, through a process called pyrolysis. Given enough time, aromatization proceeds at even lower temperatures. The aromatic hydrocarbons of crude oil are formed over millions of years in sediments that are at temperatures between 100° and 150°C.

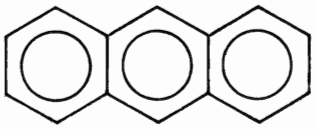
A quite different pattern is observed when the temperature of formation is lower. That pattern is best illustrated by the analysis of crude oils; there alkylated polycyclic aromatic hydrocarbons far exceed the unsubstituted ones, and the average degree of alkylation and the maximum number of carbons on the aromatic rings are far higher than they are in samples produced by high temperature pyrolysis.

It has been conjectured that polycyclic aromatic hydrocarbons are being produced today by thermal processes in soils at environmental temperatures. The isolation of benz[a]pyrene in rural soils stimulated world wide analysis. By the end of the 1960's, it was generally accepted that from 10 to 15 hydrocarbons which show an intense fluorescence in ultraviolet radiation are present in most if not all soils in similar proportions everywhere. Unsubstituted aromatic hydrocarbons are the most abundant; they are accompanied by extended

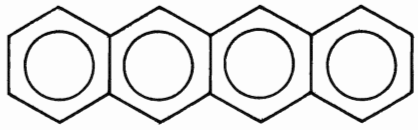
Figure 1. Environmental PAHs



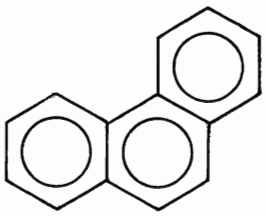
Napthalene



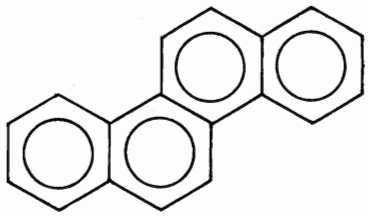
Anthracene



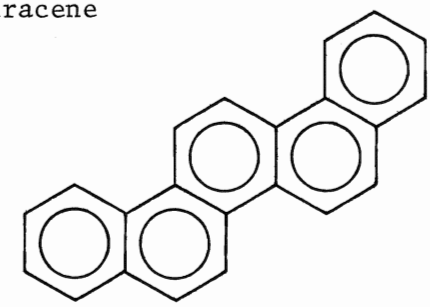
Tetracene



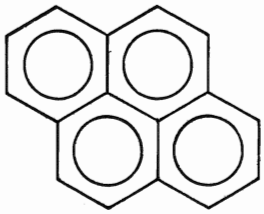
Phenanthrene



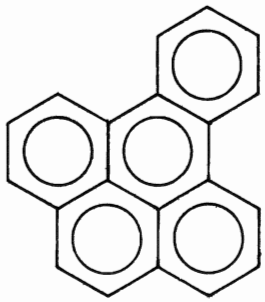
Chrysene

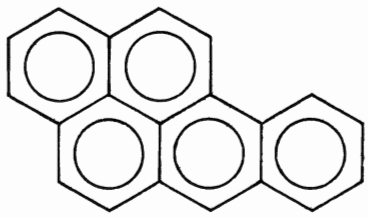


Picene

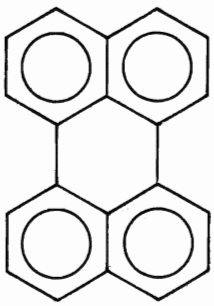


Pyrene

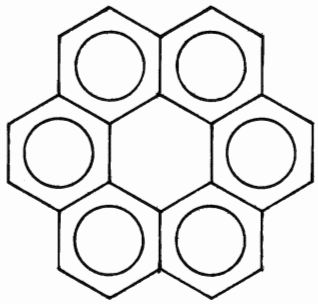




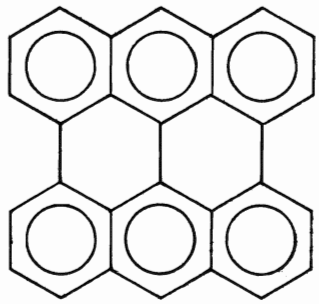
Isomeric Benzopyrenes



Perylene



Coronene



Meso-Napthodianthrene

series of substituted members containing alkyl group and saturated five-member rings.

The ring arrangements in polycyclic aromatic hydrocarbons can be divided into three broad categories (Fig. 1): linear (with all the rings in a line), angular (with the rings in steps) and clustered (with at least one ring surrounded on three sides). The various arrangements differ in their stability. The linear arrangement in anthracene and tetracene is the least stable. Clusters of benzene rings, as in pyrene, benzopyrene and perylene, are more stable and are commonly found in pyrolysates. The most stable configuration is the angular arrangement of benzene rings in phenanthrene, chrysene and picene. The last category of compounds is common in soils, sediments, wood tar, tobacco smoke, petroleum and atuomobile-exhaust gases.

PAHs from pyrolysis have been present on the earth for a long time. Human population has been chronically exposed to combustion products throughout its history, and natural fires and reactions in sediments formed polycyclic aromatic hydrocarbons long before the advent of man. Moreover, hydrocarbons in smoke, in fallout from polluted air, in sediments and fossil fuels also come in contact with man.

PAHs now enter the environment in larger amounts than they did in the early stages of evolution, and from some recently recognized sources.[2,3] The major sources include the incomplete combustion of wood, coals, the spillage of raw or refined petroleum and metal smelting plants.

Polycyclic aromatic hydrocarbons are noted for their biological effects. Some of them can induce cancer or cause mutations, even in very low concentrations. This discovery caused a great concern among scientists. Environmental chemists, geochemists and toxicologists pursued the study of identification of polycyclic aromatic hydrocarbons and their natural pathways of metabolism to assess the effects of release of these compounds into the environment. The environmental mixture of aromatic hydrocarbons is exceedingly complex, displays a wide range of cancer inducing activity[4], has many sources, and is difficult to analyze. Since many of these compounds have never been tested for their biological activity, it seems important to do a thorough investigation of the environmental effects of the new influx of these substances. The present study (in part) is a step towards the examination of these compounds in biological systems in the light of our present knowledge of the subject.

The metabolic conversion of aromatic compounds to phenols in mammals has long been considered a means for the detoxification and excretion of a variety of foreign compounds and environmental pollutants. The idea began to emerge that the biotransformation of foreign compounds into less lipid-soluble products, which would be more readily excretable from the body, might well confer a protective function.

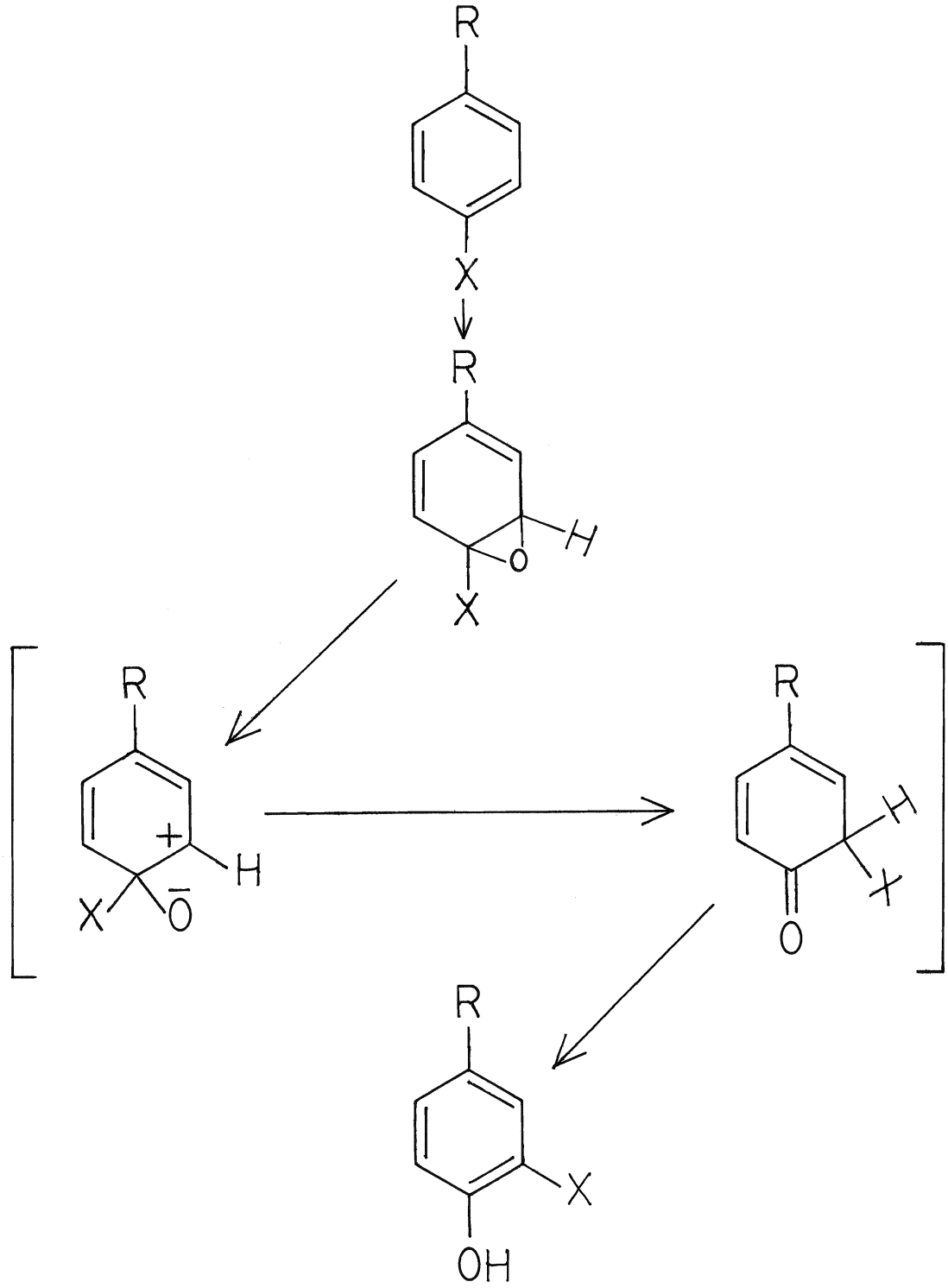
The major pathways for metabolism of polycyclic aromatic compounds in biological systems are catalysed primarily by a class of nonspecific

monooxygenases known as cytochrome P-450. Cytochrome P-450 activity is localized in the endoplasmic reticulum (microsomal fraction) of cells from liver, lung, kidney, intestine, and skin. Highest activities of P-450 are found in liver.

Recent investigations[5-7] into the mechanism by which hepatic monooxygenase enzymes catalyse the formation of phenols have, however, revealed that highly carcinogenic arene oxides, compounds in which a formal aromatic double bond has undergone epoxidation, are intermediates in the reaction. Identification of arene oxides as intermediates in the metabolic formation of phenols and the realization that the carcinogenic activity of polycyclic aromatic compounds is only manifested following their metabolism [5-7] has stimulated a great surge of interest among the researchers to exploit this new aspect of metabolism and fate of PAHs in mammalian,[8] bacterial,[9] and fungal[10-13] systems.

The migration and retention of substituents that occur during the monooxygenase-catalysed formation of phenols from most aromatic substances has been termed as the "NIH shift".[14]. The occurrence of this shift suggested that the "aromatic hydroxylations" are actually epoxidations and that the intermediate arene oxides undergo a ready isomerization to phenols with concomitant migration and retention of substituents (Fig. 2). A wide variety of substituents including isotopes of hydrogen, halogens and allyl groups have been observed to migrate in this way, and monooxygenases from plants, microorganisms, and animals catalyse the initial epoxidations.[15,16]

Figure 2. The NIH shift during the monooxygenase-catalysed formation of phenols



Arene oxides, in addition to isomerization to phenols, react readily with nucleophiles such as glutathione, and undergo enzymatic hydration (Fig. 3). The steady-state concentrations of arene oxides depend on the rates at which they are formed and their ability to undergo the above mentioned transformations.

Aromatic compounds with an oxidizable substituent, such as the methyl group in toluene, are often metabolized to a large extent at such moieties rather than by way of arene oxide formation of the ring. The relative extent of formation of different oxides and other metabolites from an aromatic substrate will, of course, depend on the biological system.

The stability of a particular arene oxide will also influence the extent of its nonenzymatic reactions with a variety of nucleophiles, including such intracellular macromolecules as DNA, RNA and protein. Virtually nothing is known of the relative stability of these isomeric oxides except that the K-region oxides are considerably more stable than the non-K-region oxides.[5] A "K-region" is defined as the external corner of a phenanthrenenic moiety in a PAH (Fig. 4). The "K-region" terminology derives from the Pullman theory[17], according to which carcinogenic polyarenes were considered to be distinguished by an electron rich bond, such as 4,5 bond of B.P., which is termed as the "K-region" and excision of which leaves a fully aromatic polyarene ring system. Direct evidence has now been provided that K-region oxides are formed as metabolic intermediates from phenanthrene,[18]

Figure 3. Metabolism of Benzene Oxide

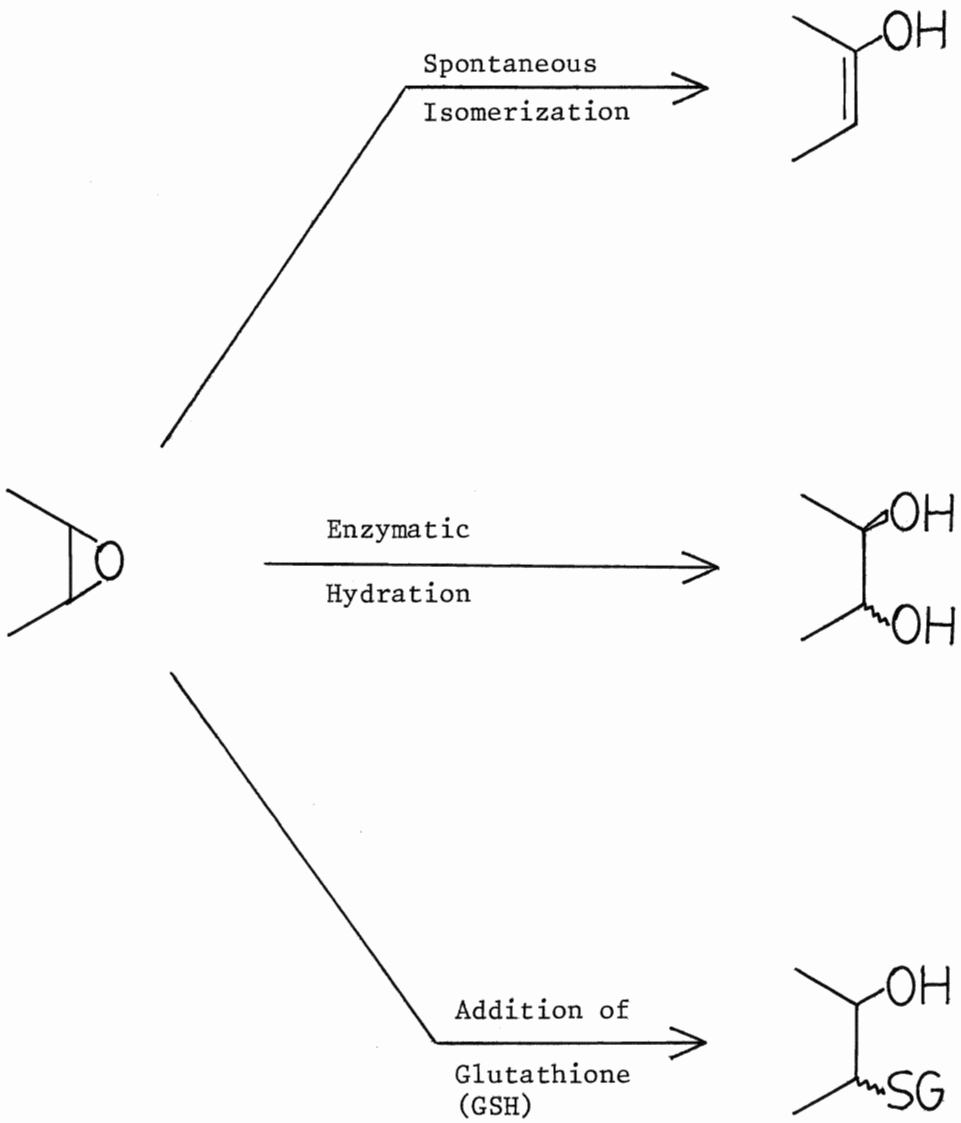
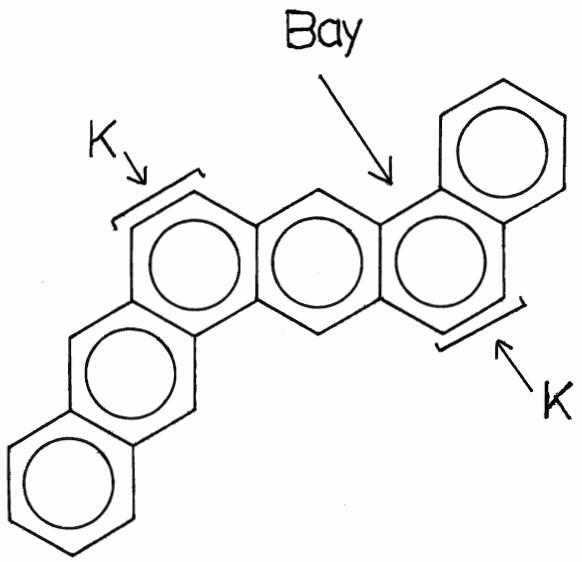


Figure 4. Possible sites for bioactivation in a PAH



benzo[a]anthracene,[18] dibenzo[a,h]]anthracene,[18,19] pyrene,[20] and benzo[a]pyrene[20,21] in the 4,5 position (Fig. 5).

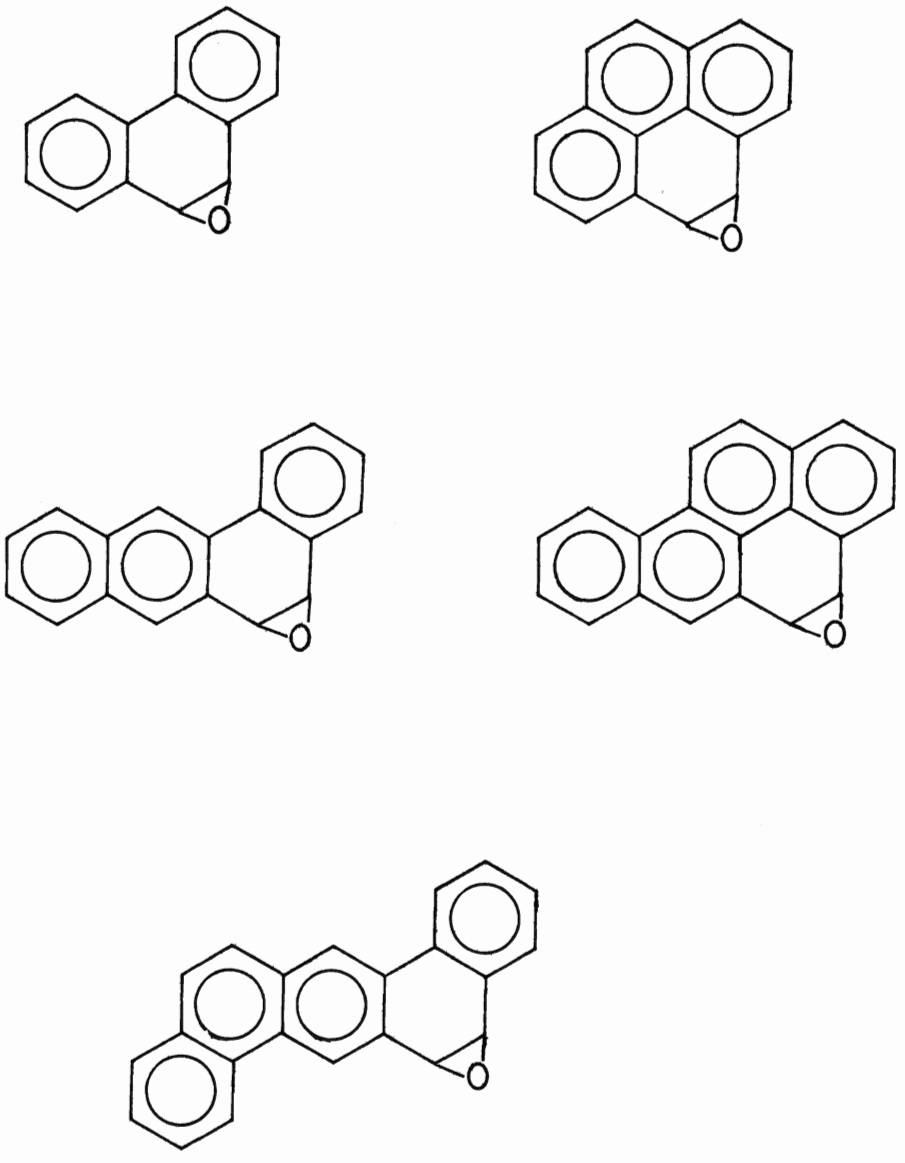
Detailed investigations[22,23] of the mechanism of isomerization have indicated that carbonium ions (Fig. 2) are formed during the rate determining steps in these reactions. The formation of phenol depends upon the relative stabilization of such transient carbonium ion intermediates. In certain instances, these carbonium ions are readily trapped by reaction with solvent molecules.[24,25] This facile solvolysis is indicative of how readily arene oxides should be able to bind even to very weak nucleophilic sites within the cell. On the other hand, the results from the addition of glutathione indicate that nucleophilic opening of the oxide can take place, rather than trapping of an intermediate carbonium ion.

The complexity of the reactions of arene oxides with simple nucleophiles indicate that reactions with DNA, RNA and protein will in all likelihood proceed by many pathways.

A variety of chemicals have been implicated as causative agents in carcinogenesis. Each of the chemical caracinogens that has been thoroughly studied has been found to bind covalently with DNA, RNA and protein of the target tissue.[26]

However, PAHs do not covalently bind to biopolymers without prior specific bioactivation; presumably a monooxygenase, cytochrome P-450 catalysed conversion to arene oxide. The magnitude of the weak physical

Figure 5. K-region oxides that have been identified as metabolites of PAHs



binding which occurs between the hydrocarbons and macromolecules does not correlate with the carcinogenic efficacy of the hydrocarbon.[27] Thus, arene oxides have become prime candidates for the bioactivated intermediates responsible for the covalent bonding of aromatic compounds to biopolymers within the cell.[28-31] Since the toxic, carcinogenic, and mutagenic effects of PAHs often correlate with the extent of this bonding, it provides a molecular basis for these effects and reveals arene oxides as causative agents in producing these effects. Covalent binding alone, however, cannot be considered a sufficient criterion for carcinogenesis; benz[a,c]anthracene and the [a,h] isomer bind equally well to mouse skin, yet only the latter compound is carcinogenic.[32]

The proposal that arene oxides are the bioactivated intermediates responsible for the binding and the carcinogenic effects of PAHs is attractive. However, the possibility that metabolites other than arene oxides also have a role in the carcinogenicity should certainly not be excluded.

Some studies did not implicate arene oxides as the ultimate carcinogens formed from PAHs. Carcinogenicity tests show the arene oxides to be generally less active than the parent hydrocarbons.[33,34] While activated metabolites might be expected to exhibit greater activity than their precursors, these negative results, however, may be the consequence of transport and/or inactivation phenomena, due to decomposition or secondary reaction of these oxides in the intact organism prior to reaching the critical target macromolecule.

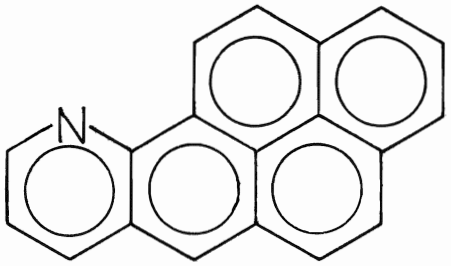
Although these studies have been highly informative, questions such as whether or not K-region oxides, non-K-region oxides, or subsequent metabolites are the ultimate carcinogens or mutagens remain to be answered, perhaps through careful parallel comparisons of biochemical formation and disposition of hydrocarabon metabolites and their biological effects. One of the biochemical parameter of relevance is the nature and extent of binding of metabolically active PAHs and of their various derivatives to biopolymers.

The nature of the sites of binding to biopolymers and the complex set of kinetic and structural parameters that influences both the reactivity of arene oxides and their ability to bind to critical target molecules is under investigation, but such factors have not been completely defined.

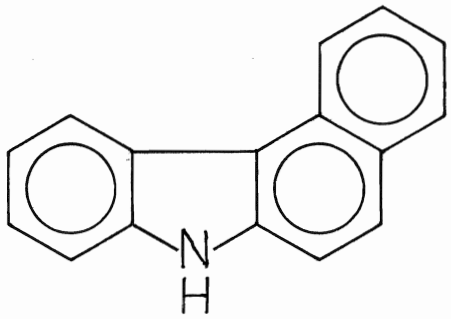
Throughout the development of this field of knowledge, the operation of biochemical reaction had been assumed, and the establishment of enzyme-catalysed mechanisms for the reaction of foreign compounds and for intermediate metabolites strongly supported this supposition. Nevertheless, review of the literature[35] indicated some metabolites, which were unlikely to result from biotransformations alone. Non-enzymatic reactions contribute more extensively[36] to the metabolism of foreign compounds in mammals than is generally supposed. Thus, this aspect of chemical transformations in biological systems can not be ignored completely during the investigation of the metabolic fate of PAHs in these systems.

A large effort has been made to the study of PAHs and their role as potent carcinogens in biological systems. On the other hand, such studies of heteroanalogs of PAHs are quite poor. However, the presence of aza, thia and oxa aromatics as environmental atmospheric pollutants has recently been recognized.[37-39]. Polycylic aromatic compounds (PACs) as distinct from the hydrocarbons (PAHs) are formed from both natural and anthropogenic sources; the latter are by far the major contributors. Natural sources include forest fires, volcanoes and synthesis from degraded biological substances. Recently, Kosuge *et al* [39] reported the isolation of five nitrogen containing PACs from basic fractions of coal tar and all of them appeared to be mutagens (Fig. 6). Major anthropogenic sources include the burning of coal, residential fireplaces, automobile exhausts, coal-fired residential furnaces, oil-fired commercial boilers, commercial incinerators and rubber tyre wear. A minor source in terms of total PAC production, but of considerable importance with respect to human health, is tobacco smoking. Until the beginning of this century there existed a natural balance between the production and natural degradation of PACs, which kept the background concentration low and fixed. However, with increasing industrial development throughout the world, the natural balance has been disturbed and the production and accumulation rates of PACs are constantly increasing. These compounds have also been identified as components of urban air particles. Because of their distribution, they may represent an inhalation hazard in both the occupational and general environment.

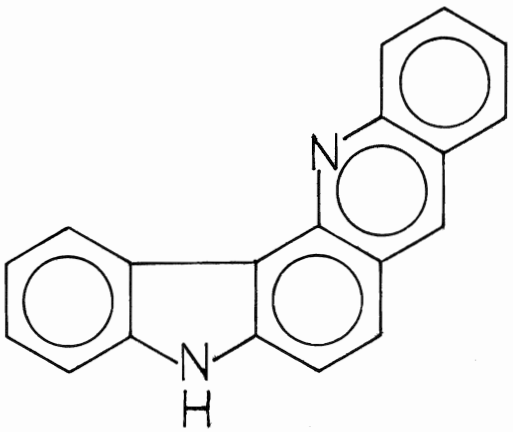
Figure 6. Mutagens isolated from the basic fraction of coal tar



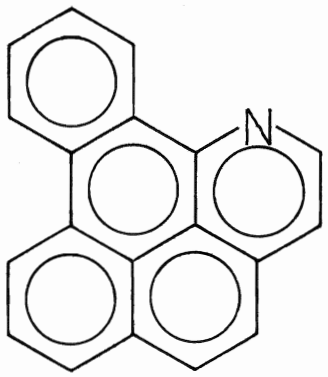
Phenaleno[1,9-gh]quinoline



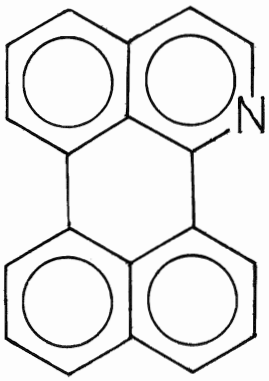
Pyrido[3,2-c]carbazole



Quino[3,2-c]carbazole



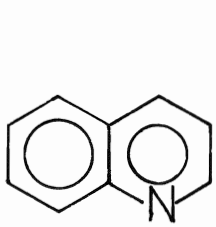
Benzo[h]naptho[2,1,8-def]-quinoline



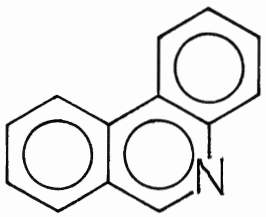
Benzo[de]naptho[1,8-gh]quinoline

In view of demonstrated carcinogenic or mutagenic behaviour of many PACs (Fig. 6 and 7), efforts had been made to characterize these compounds in various environmental sources, with which human population may come in contact or to which they might be exposed. In contrast studies on the metabolism of such compounds in biological systems are limited.[39-43] However, the preliminary results suggested a relationship between the biochemical metabolic pathways of PAHs and their heteroanalogs in the sense that the latter also require "bioactivation" catalysed by monooxygenase cytochrome P-450 to show mutagenic and carcinogenic effects.[40,44] The bioactivated metabolites are presumably those forms which ultimately attack critical cellular components, possibly nucleic acids. In particular, oxidation reactions on angular benzo rings, leading ultimately to so-called "bay region" dihydrodiol epoxide and carbonium ion forms (Fig. 8) appear to be central to carcinogenic activation in PACs. The term "bay region" refers to concave, exterior topological region of the hydrocarbon bordered by three benzene rings, one of which must be a terminal ring. The typical example of bay region is the sterically hindered area between the 1 and 12 positions in benzacridines (Fig. 7). Experiments with benzacridines[42] indicated epoxidation and hydrolysis of the epoxides to dihydrodiols to be the predominant metabolic pathways. However, no unequivocal evidence could be obtained for the formation of ultimate carcinogen, the 3,4 dihydrodiol-1,2-epoxides in case of the

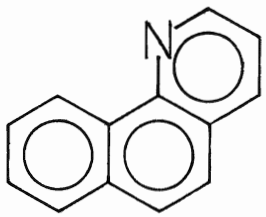
Figure 7. Carcinogenic PACs



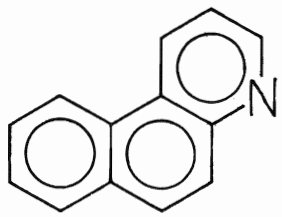
Quinoline



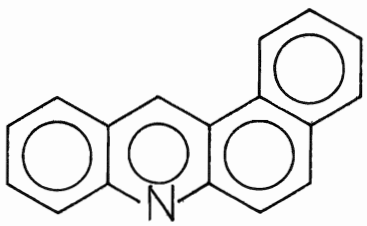
Phenanthridine



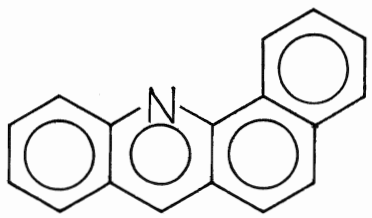
Benzo[h]quinoline



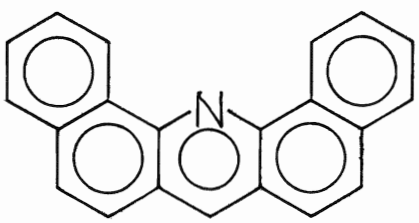
Benzo[f]quinoline



Benz[a]acridine

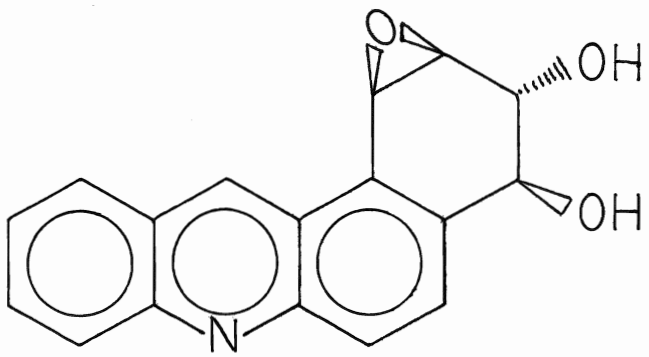


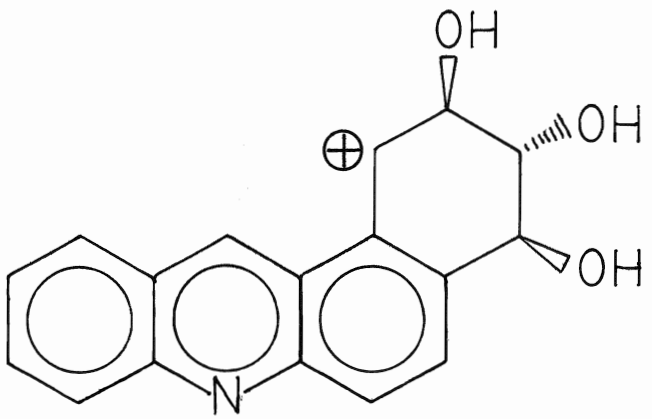
Benz[c]acridine



Dibenz[c,h]acridine

Figure 8. Possible bioactivated metabolites of benz[a]acridine





benz[a] and benz[c]acridine.

Further detailed studies regarding the structural requirements and mutagenic and carcinogenic activity of putative metabolites of polycyclic heteroaromatic compounds are in progress.[45-48]

Very little is known about the properties of polycyclic heteroaromatic compounds, although numerous aza-PAH are carcinogens and are known to be environmental contaminants present in urban atmosphere, tobacco smoke and automobile exhausts. Several different bacterial and fungal species which may possess the capabilities to metabolize these compounds have been isolated from terrestrial and aquatic environments. It therefore is of interest to study the metabolite profiles of these compounds; this aspect of investigation is of great environmental importance in that it provides knowledge about the *in vivo* formation of highly carcinogenic, mutagenic and toxic arene oxide metabolites in the natural environment (soil, water, air etc.) of the microorganisms concerned.

Recent research in this fast-developing field have shown *trans* dihydrodiol epoxide to be the activated mutagenic form of certain PAHs.[49] Other *in vivo* / *in vitro* studies [50-55] have revealed the adduct formation of these putative metabolites and/or their precursors with DNA and/or RNA. In most cases, the adduct formation through covalent bonding has been indicated.

One of the very interesting and important observations made in the last few years by Cerniglia, Gibson and Morgan[56,57] is that some eucaryotic microbes, e.g., *Cunninghamella elegans* use

mono-oxygenative enzymic mechanisms for the ring attack of polycyclic aromatic compounds, as do the mammals, to initiate detoxification reactions; arene oxides can undergo isomerization to yield phenols and can also serve as substrate for the enzyme epoxide hydratase which catalyses the addition of water to yield trans-dihydrodiols. In contrast, bacteria utilize a dioxygenase enzyme system that incorporates both atoms of molecular oxygen into the substrate to form cis-dihydrodiols (Fig. 9). In another study,[58] bacterial oxidation of PAHs to cis-dihydrodiols has been shown. It can be seen that the determination of the relative stereochemistry of dihydrodiols formed by different biological systems is important since such information provides evidence for the reaction mechanisms utilized in the enzymic oxygenation of aromatic hydrocarbons.

Mammalian enzymes involved in the metabolism of aromatic compounds have been subjected to intensive investigations. It is now known that the oxidation and subsequent elimination of aromatic hydrocarbons from animals occur through reactions mediated by cytochrome P-450,[59] epoxide hydrolase,[60] glutathione S-transferase[60] and UDP-glucuronosyltransferase[61,62] (fig. 10).

In contrast, information about the presence or function of these enzymes in eukaryotic micro-organisms is limited. However, cytochrome P-450 has been detected in several fungi.[63-65] In the preliminary studies by Ferris and co-workers[63,66] using Cunninghamella bainieri , it was observed that the hydroxylation of 4-[^{2}H]-anisole

Figure 9. Metabolic pathways utilized by eucaryotic and procaryotic organisms for the oxidation of aromatic hydrocarbons. The dioxetane intermediate shown has not been formally demonstrated.

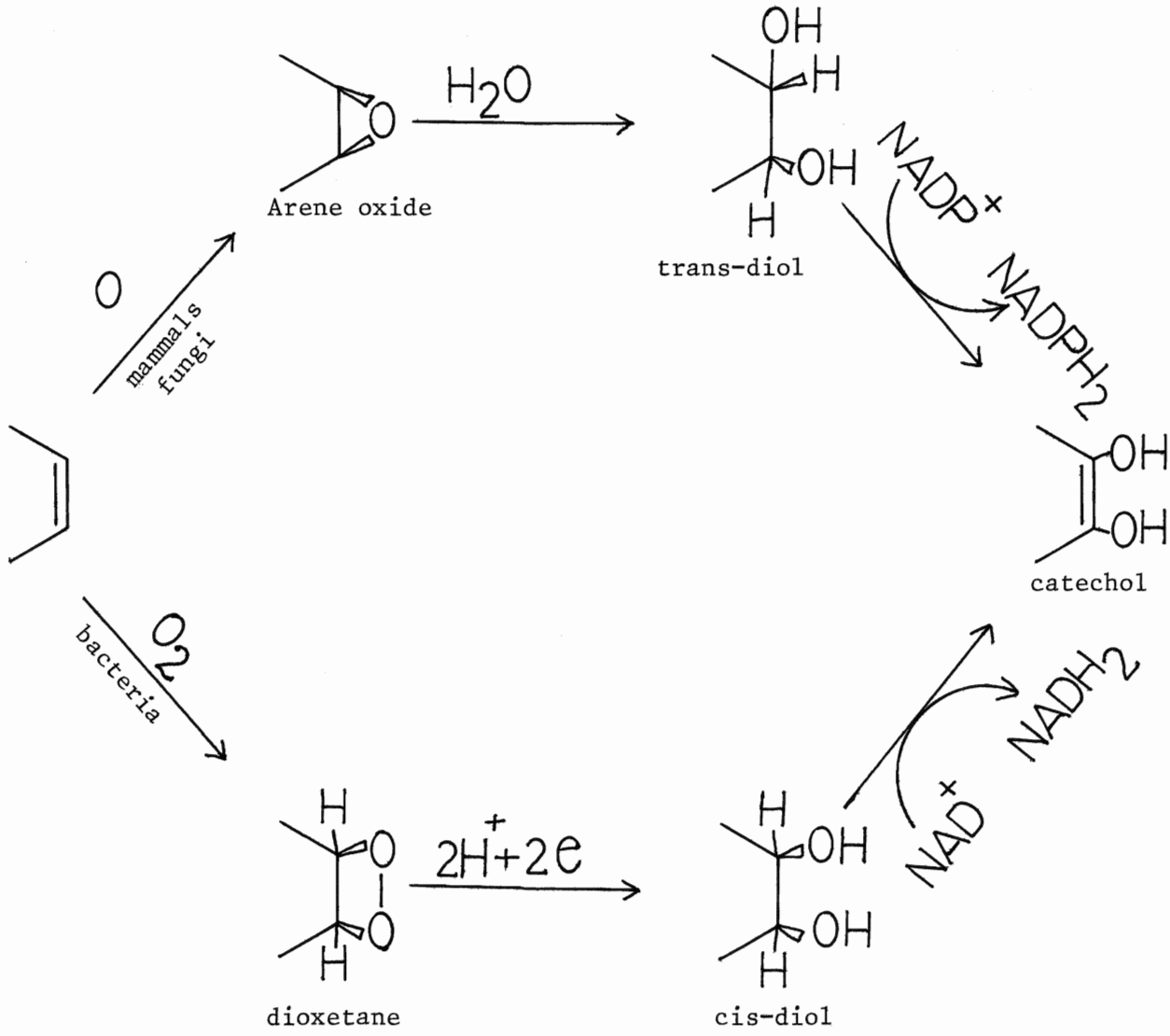
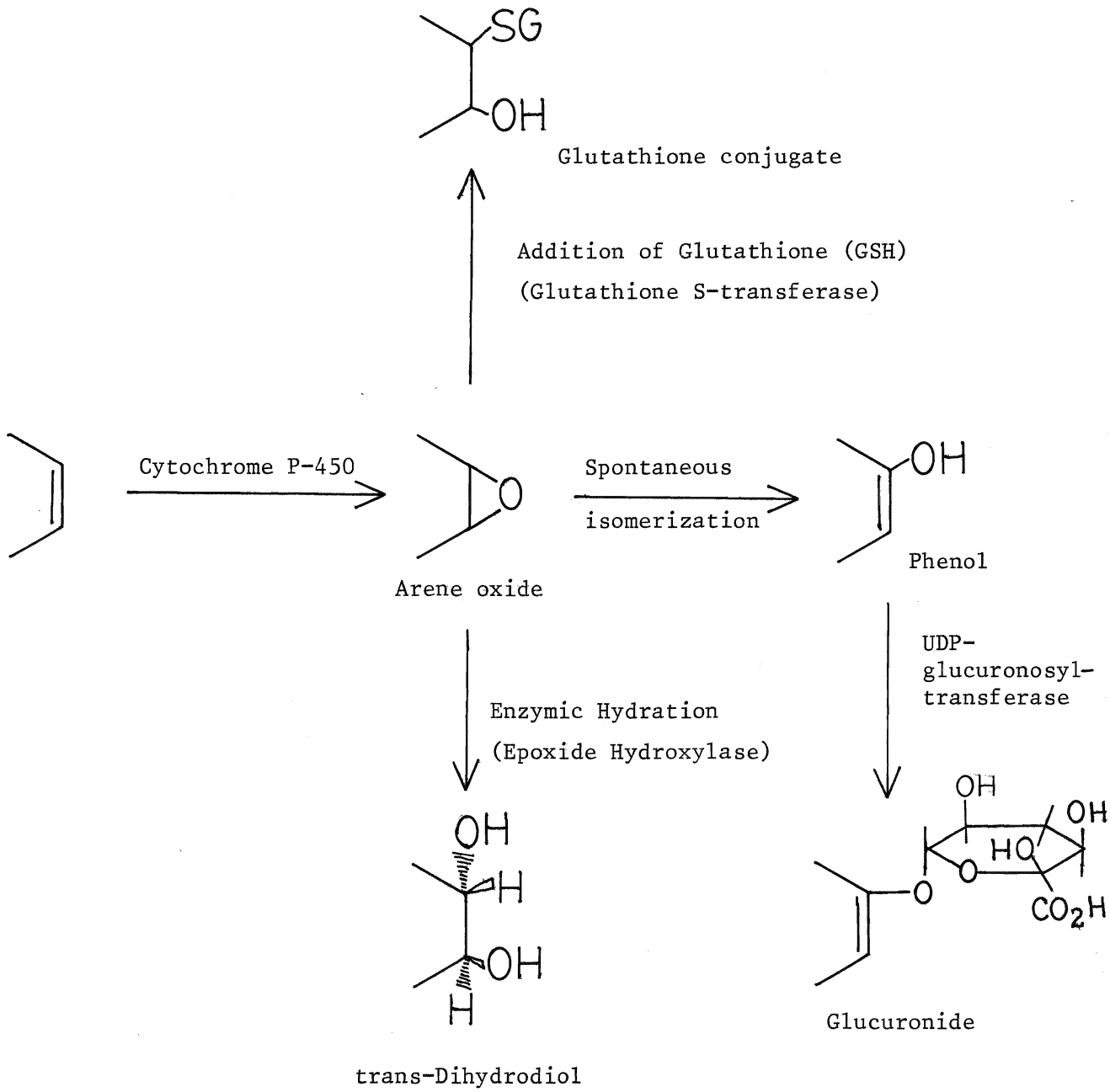


Figure 10. Reactions utilized by mammals for the metabolism of PAHs



and 2-[^{2}H]-anisole proceeded with migration and retention of isotopic hydrogen (NIH shift). The formation of the trans-dihydrodiol of the naphthalene and the incorporation of only one atom of oxygen-18 from $^{18}O_2$ into the trans-dihydrodiol are consistent with naphthalene oxide as an intermediate and the presence of an epoxide hydrase in C. bainieri. The product ratios in these hydroxylations were very similar to those obtained using liver microsomes. C. bainieri also metabolises aniline by aryl hydroxylation, aminopyrine by O-demethylation, and nitro- and azo- compounds by reduction under anaerobic conditions; these transformations are all typical of reactions catalysed by liver microsomes. This evidence suggests that there is a close similarity between fungal and mammalian cyclochrome P-450. The enzymic activity in the fungi is inhibited by CO, SKF 525-A and metyrapone, but not cyanide. These data together with the pattern of stimulation by metal ions, suggest that fungal enzymatic activity is due to a cytochrome P-450, similar to the one present in mammalian liver. The conclusion was confirmed when a maximum absorbance at 450 nm was observed in the difference spectrum of the fungal hydroxylase after reduction with dithionite and addition of CO.

In another study, Gibson et al [64] reported that the metabolism of naphthalene by crude microsomal preparations from C. elegans is very similar to that observed with hepatic microsomes and reconstituted enzyme systems that contain partially purified preparations of cytochrome P-450. This conclusion was based on the observations that

specific cytochrome P-450 inhibitors, carbon monoxide, SKF-525-A and metyrapone, reduced the formation of *trans*-naphthalene dihydrodiol and 1-napthol by the microsomal preparations. The insensitivity of the system to cyanide is also a property of cytochrome P-450. These results are similar to those reported for enzymic activity in the related organism *C. bainieri.*

The level of cytochrome P-450 in the microsomal preparations of *C. elegans* was much lower than that in hepatic microsomes, but was quite similar to the level found in *C. bainieri.*

Recent studies[64,67] have demonstrated that *C. elegans* contains epoxide hydrolase, glutathione S-transferase and UDP-glucuronosyltransferase activities which share several properties with the same enzymes found in mammalian liver.

In summary, *C. elegans* contains enzyme activities involved in the transformation of polycyclic aromatic compounds that are similar to those found in mammals. Further investigations will be required to determine whether these enzymes participate in essential metabolic reactions or are present to protect the fungus from the effects of environmental chemicals.

A screening programme[10] revealed that forty seven species of fungi belonging to thirty-four genera from the major fungal taxa have the ability to metabolise naphthalene. The predominant metabolite formed by most organisms was 1-naphthol. *Trans* -1,2-dihydroxy-1,2-dihydronaphthalene was also identified among other

products. The fungi surveyed include organisms that are found in terrestrial, freshwater and marine ecosystems.

Thorough survey of the literature has shown remarkable similarities between the metabolism of aromatic compounds by mammals and many fungal species. For example, NIH shift and incorporation of one atom of molecular oxygen during metabolism of various substrates with a range of fungi,[66,68,69] broad spectrum activity and the inhibition plus stimulation shown by C. bainieri ,[63,66] C. elegans oxidizes naphthalene,[56] anthracene,[70] biphenyl,[71] benzo[a]pyrene,[11] and 3-methylcholanthrene to complex mixtures of hydroxylated products that are remarkably similar to those produced by mammalian liver. A cytochrome P-450 monooxygenase[63,64] has already been detected and recently the presence of enzyme epoxide hydrolase,[64,67] glutathione S-transferase,[67] and UDP-glucuronosyltransferase[67] activity similar to hepatic microsomes has been established in many fungi. The results suggest definite similarities between the enzyme systems used by fungi and cytochrome P-450 dependent mono-oxygenase activity that is associated with mammalian liver microsomes.

Now the question arises whether the fungi can be used as a model of mammalian metabolism. The knowledge we obtained in this field so far clearly indicates that it might be possible to define a microbial transformation system which would mimic many types of biotransformations reported to occur in mammals. Such a microbial transformation system,

consisting of a selected series of microorganisms, could advantageously be used to conduct parallel mammalian and microbial studies of foreign compound metabolism. When metabolites common to both systems are observed, the microbial system would be utilized in obtaining sizable quantities of metabolites *via* routine incubation techniques. By this process, difficult to synthesize metabolites could be easily obtained for structure elucidation and biological testing. The preparative scale capacity of fungal incubations as well as experimental reproducibility guaranteed by the availability of standardized cultures offer immediate advantages over mammalian systems.

To test the ease of using microbial systems to mimic mammalian metabolic patterns, attention has been focussed on a single type of reaction, aromatic hydroxylation. This particular reaction was selected for several reasons: first, it is the most common mammalian biotransformation; second, aryl hydroxylase activity similar to that displaced by mammalian cytochrome P-450 has been demonstrated in several microbial species; third, phenol formation takes place *via* arene oxide intermediate which is recognized as an immediate carcinogenic metabolite of PAHs.

Rosazza and Smith,[72-75] have exploited this interesting aspect of great importance through a series of experiments. They chose micro-organisms from among a wide variety of generae of fungi and did the incubations with various model compounds varying in functionalities. In most instances, the microbial model system gave good qualitative

correlations with reported metabolic profiles of hepatic microsomes and/or *in vivo* mammalian systems.

Although some metabolites were estimated to occur in low overall yields, it should be emphasized that it is usually possible to improve yields significantly by adjusting a number of incubation parameters to obtain optimum conditions.

Minor mammalian metabolites were often found as major metabolites of several fungi. However, with most compounds, one or more fungi consistently demonstrated the ability to produce major mammalian metabolites, thus confirming the idea that fungi may also serve as useful models for the study of foreign compound metabolism in mammals.

Clearly, further studies are required to prove the versatility of the present hypothesis. Nevertheless, the information revealed by these experiments is of great value in understanding the metabolic pathways in mammals and ultimately in man.

The present study (in part) explores the fate of polycyclic aromatic compounds by fungal metabolism. Interest in the metabolism of PAHs and their heteroanalogs by fungi stems from the fact that in addition to their importance as widespread environmental organisms, many fungi are reported to possess metabolic capabilities which often closely relate those of mammalian hepatic microsomes, and so fungal systems are being used as a model for mammalian metabolism.

PART TWO

Microbial Oxidation of Alkylbenzenes

In addition to the metabolism of various PAHs and their heteroanalogs, the ability of cytochrome P-450 containing systems from microbial and mammalian sources to oxidise the simple hydrocarbons was recognized a long time ago, and since then these compounds have been the subject of research. The substrates for this type of oxidation range from the long chain aliphatic hydrocarbons to substituted alkylbenzenes.

The distribution of isomeric alcohols, formed from alkanes by the action of mammalian liver monooxygenase, has been measured.[76] All of the possible isomeric alcohols were formed from methylcyclohexane, 2-methylbutane, 2,2-dimethylpropane, isobutane, n-pentane and n-butane, except for the primary alcohols of the latter three substrates. Common features were the high degree of hydroxylation at secondary, relative to primary, C-H bonds, and the preferred hydroxylation of tertiary C-H bonds. The small size and lack of functional groups in these molecules probably leads to no rigid orientation at the site of hydroxylation, and product ratios could be governed by the reactivities of the various C-H bonds. The specific activity of the enzyme was similar toward all substrates, and was induced four to fivefold on pretreatment of rats with phenobarbital; the binding of the substrate to cytochrome P-450 also increased with the induction of enzyme activity.

n-Hexane is also hydroxylated by liver microsomes in all positions. The preference is for 2-hydroxylation, but this is due to steric

factors, since the 2- and 3-positions are almost equivalent in chemical reactivity. Phenobarbital pretreatment induces 2- and 3-hydroxylation sixfold; 3,4-benzpyrene suppresses 2- and stimulates 3-hydroxylation. The inhibitors, metyrapone and 7,8-benzoflavone, have a differential action on 3-hydroxylation.[77] Rat liver microsomal mono-oxygenase also catalyse the hydroxylation of n-heptane to yield all the isomeric alcohols, 2-heptanol being the main product.[78] Interestingly, although the C-H bond strengths at C-2, C-3 and C-4 are similar, attack at C-3 and C-4 is relatively smaller. Pretreatment of rats with phenobarbital causes a four-fold increase in 2-, 3- and 4-hydroxylation, but a relatively small increase in 1-hydroxylation. Benzpyrene pretreatment causes a dramatic change in relative proportions of different alcohols. Inhibition by carbon monoxide and metyrapone also affects 1- and 2-hydroxylation differently. These results suggest that more than one enzyme is involved in these reactions.

Alkylbenzenes provide options for pathways of biotransformation alternative to the direct oxidation of side chain as in the case of metabolism of alkanes. Thus the aromatic nucleus can be oxidized prior to side chain hydroxylation, or it can be retained to give homologues of alkane metabolism.

Kitagama[79] showed that when suspensions of Pseudomonas aeruginosa were exposed to toluene, enzymes for the oxidation of toluene, benzyl alcohol, benzaldehyde, benzoate and catechol were induced. This suggested that the methyl group of toluene was oxidised

to benzoate prior to the formation of the ring-cleavage substrate, catechol, presumably via the carboxyhydrodiol described later by Reiner and Hegeman[80] (Fig. 11).

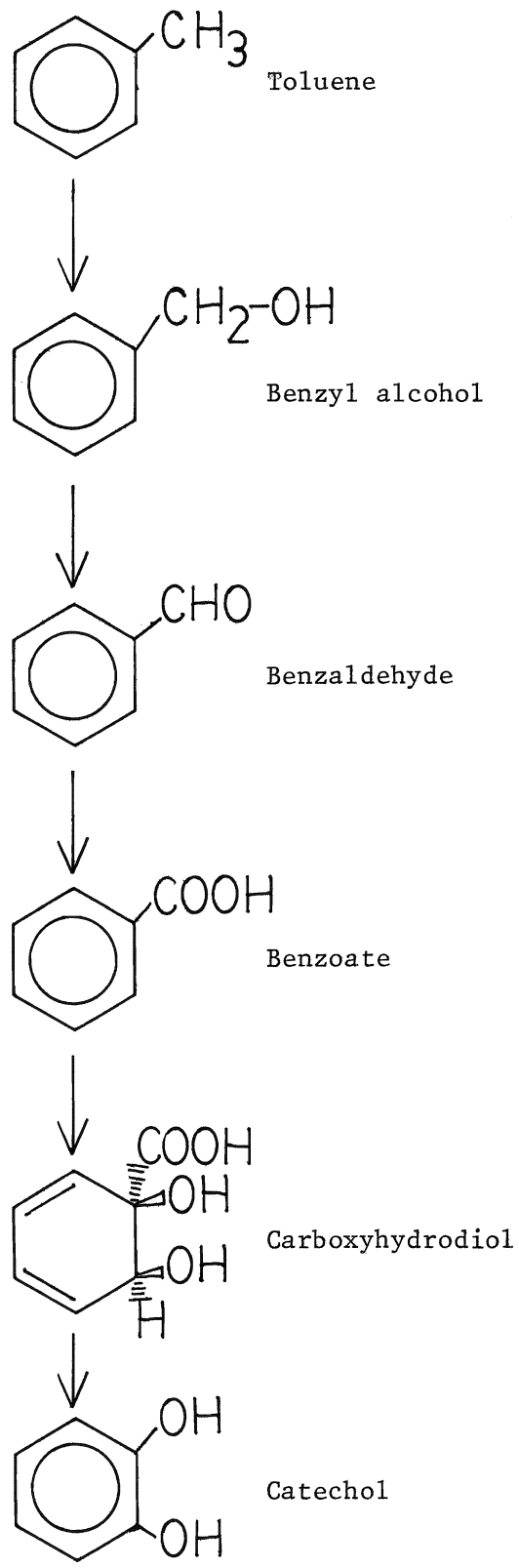
Davey and Gibson[81] looked into the bacterial metabolism of para and meta xylene and found that various strains of Pseudomonas were capable of performing successive single step oxidations of one methyl group to form the respective toluic acids. The authors proposed the pathways for conversion of toluic acid to the respective methyl catechols similar to those shown in Figure 11.

The bacterial metabolism of p- and m-xylene may also be initiated by direct enzymatic oxidation of the aromatic nucleus.[82]

The metabolism of ethylbenzene has been known as early as 1901.[83] Both the in vivo and in vitro oxidation of this substrate by mammals as well as by microorgaznisms is well documented in the literature.

Smith and coworkers[84] discovered that an injection of ethyl benzene into rabbits resulted in the urinary extraction of 1-phenylethanol. Since the formation of 1-phenylethanol involves the production of a chiral centre at carbon atom, the stereochemical implications of the oxidation of ethylbenzene became important. The same authors[85] reported that ethylbenzene is hydroxylated in the rabbit to both stereoisomers of 1-phenylethanol in equal amounts, whereas acetophenone is reduced to only one form, namely (-)-1-phenylethanol.

Figure 11. Pathways for Toluene metabolism



Many tentative mechanisms were proposed[85] for the biological hydroxylation of ethylbenzene. One possible path was *via* styrene and acetophenone (Fig. 12 a). This was not of any significance, since styrene does not yield 1-phenylethanol and acetophenone yields only the (-) isomer. Then, another alternate path involving styrene epoxide was proposed (Fig. 12 b). This proposal was mainly based on the fact that styrene epoxide can be reduced chemically to 1-phenylethanol by lithium aluminum hydride. However, styrene epoxide was not able to yield this alcohol in the rabbit and therefore it was discarded as a possible intermediate in the hydroxylation of ethylbenzene.

Since neither acetophenone nor styrene nor styrene epoxide were intermediates in the formation of (+)-1-phenylethanol from ethylbenzene *in vivo*, the authors finally suggested[85] a mechanism for the reaction in which hydrogen is abstracted from the reactive α-methylene group by an enzyme, leaving a free radical or carbonium ion. This could then combine with $\dot{O}H$ or OH^-, respectively, to form 1-phenylethanol, which would be expected to be racemic, since tervalent-carbon free radicals are planar, and when a trivalent-covalent carbon atom carries a positive charge, the ligands are coplanar (Fig. 13). They supported their arguments by the fact that the oxidation of ethylbenzene to 1-phenylethanol by a free radical mechanism has been shown to occur in purely chemical systems.

On the other hand, similar experiments[86] conducted with hepatic microsomal preparations were shown to be stereochemically selective,

Figure 12. Proposed mechanisms for Ethylbenzene hydroxylation in rats

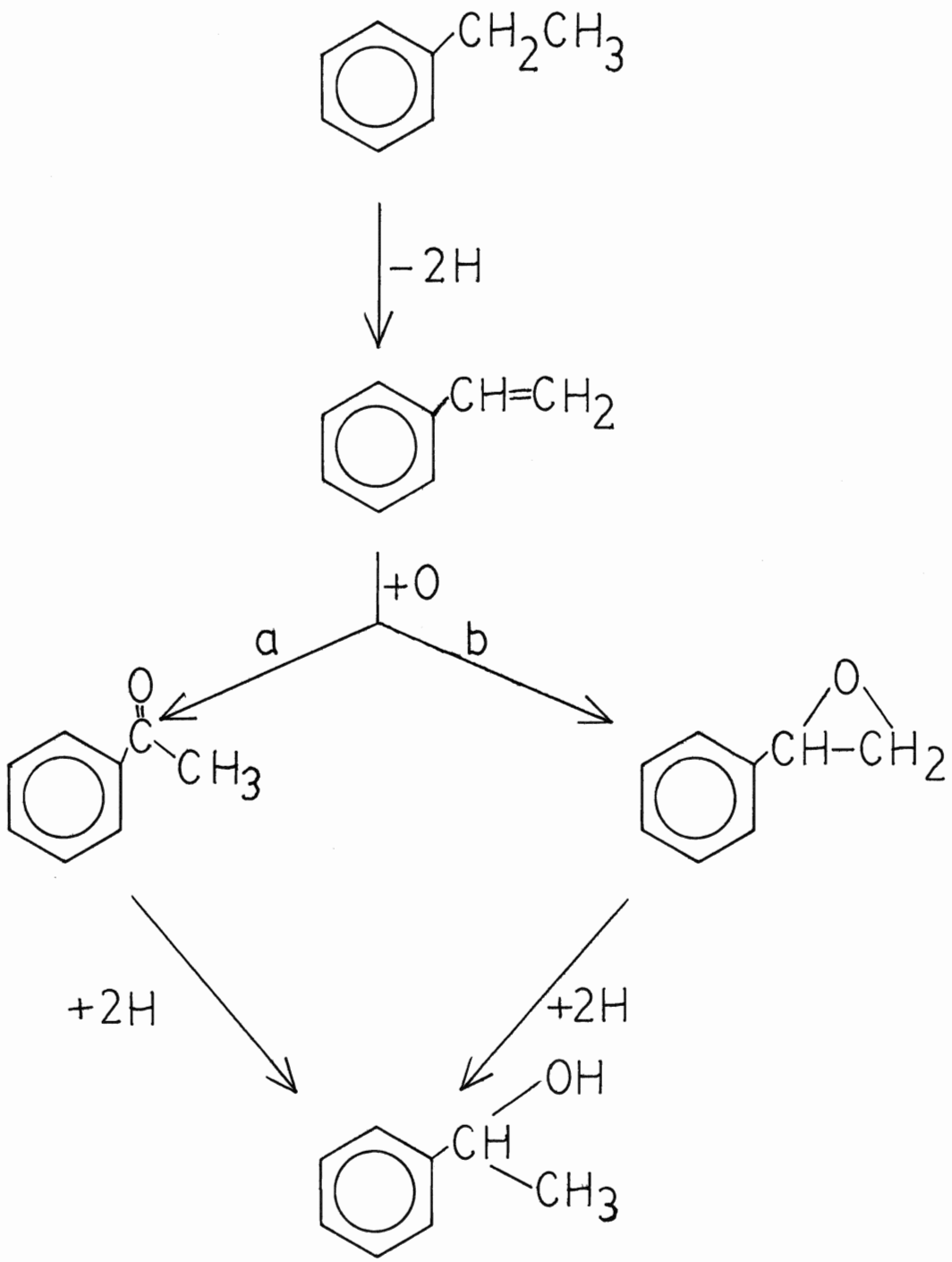
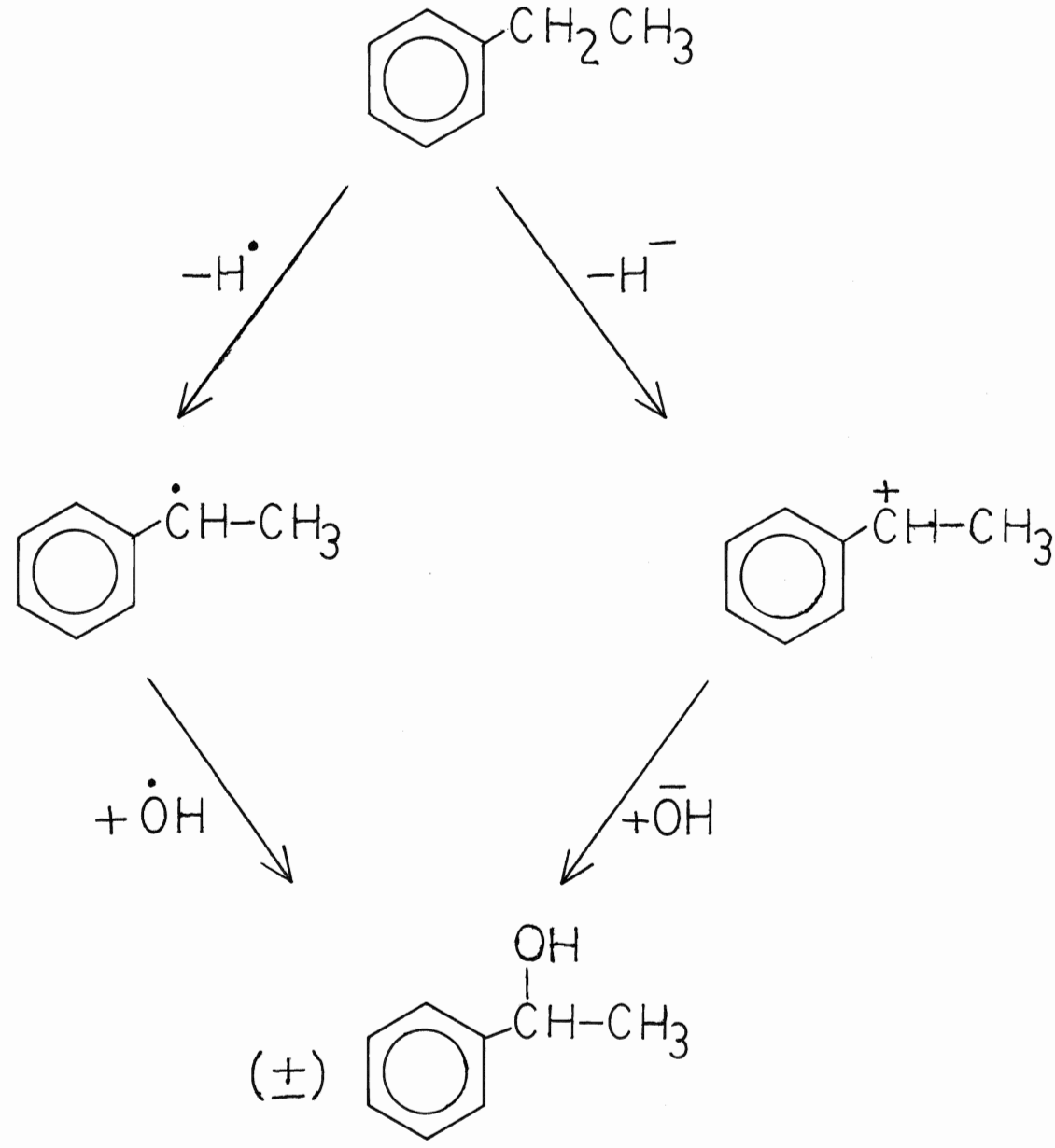


Figure 13. Proposed mechanisms for Ethylbenzene hydroxylation in rats



producing about 80% R(+)- and 20% S(-)-1-phenylethanol. Other studies[87] with rat liver microsomes produced 1-phenylethanol with S(-) isomer as a major product (ca. 80%). Further investigations[88] also revealed that S(-)-1-phenylethanol was the major product.

Further research[89] on ethylbenzene oxidation by microsomal preparations from rat liver has been carried out in an atmosphere containing oxygen-18. The oxygen-18 content of the 1-phenyl alcohol formed was estimated to be 68%, thus indicating that the hydroxylation is a true oxygenation utilizing molecular oxygen and not oxygen from water. That hydrogen from solvent is not involved was also shown. Thus, when the incubation was carried out in deuterium oxide, no isotopic hydrogen appeared in the enzymatic product.

The hydroxylation of α,α-d_2-ethylbenzene ($PhCD_2CH_3$) has also been investigated.[89] 1-Phenylethanol formation from this substrate was substantially slower due to a deuterium isotope effect which was found to be $k_H/k_D = 1.8$. The resulting alcohol retained one of the two deuterium atoms present in the starting material. The optical purity of the alcohol formed was 81% R(+), which was in good agreement with the value of 80% R(+) for alcohol obtained from the incubation of unlabelled substrate.[86] These results clearly suggested that the microsomal hydroxylation proceded by the direct replacement of one of the α-hydrogens of ethylbenzene by oxygen. In the same study, incubation of optically active S(+)-d-ethylbenzene with microsomal oxygenase system provided 91.7% R(+)-α

-d-1-phenylethanol with 86% retention of deuterium. From these results it was clear that the microsomal hydroxylation of ethylbenzene occurs with retention of configuration or, in other words, oxygenation occurs by front side displacement.

The effect of microsomal enzyme inducing agents on the stereochemical nature of hydroxylation has also been studied.[89] The results with ethylbenzene and α,α-d_2-ethylbenzene as substrates indicated that phenobarbital pretreatment substantially diminishes the stereoselectivity of microsomal hydroxylation of substrates used in the study. The authors proposed two possibilities for this effect, that enzyme formed under the influence of phenobarbital differs qualitatively from normal enzyme or there may be more than one enzyme catalyzing the transformation and these enzymes may differ with respect to stereoselectivity and sensitivity to phenobarbital induction.

In addition to 1-phenylethanol, rats also oxidise ethylbenzene to 4-ethylphenol.[90] Rat liver microsomes produce 4-ethylphenol and 2-ethylphenol.[91] It seems probable that these phenolic metabolites are formed by isomerization of the corresponding arene oxides. This aspect of metabolism has already been discussed in detail in a previous chapter.

In contrast, few published data are available pertaining to the metabolism of ethylbenzene by microorganisms that can use this hydrocarbon as its sole source of carbon and energy. Davis and Raymond[92] grew a Nocardia species on octadecane in the presence of

ethylbenzene. Under these conditions, the aromatic hydrocarbon was converted to phenylacetic acid which was resistant to further degradation. A Pseudomonas strain[93] oxidizes p-diethylbenzene exclusively to the monocarboxylic acid, p-ethylphenylacetic acid. Deuterium labelled p-diethylbenzenes show an isotope effect of $k_H/k_D = 1.7$ (Fig. 14 A). Retention of the deuterium on the carbon (Fig. 14 B) indicated that the major pathway of oxidation involves direct terminal oxidation of the side chain and not dehydrogenation followed by hydration.

The oxidation of ethylbenzene by Pseudomonas putida was reported by Gibson et al[94] and a detailed study was made. The data obtained indicated that P. putida metabolizes ethylbenzene by pathway A or B (Fig. 15). Pathway A resulted in the isolation of 3-ethylcatechol. A minor diol product of ethylbenzene oxidation (about 2%), which was more polar than the diol described, was identified from pathway B. This metabolite had also been oxidized at the benzylic carbon providing (+)-cis-3-)1'-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol. There was evidence to demonstrate that this compound was formed by the stereoselective oxidation of ethylbenzene to (+)-1-phenylethanol followed by dioxygenation of the aromatic ring, since the ethylbenzene diol did not serve as a precursor of this product whereas 1-phenylethanol formed the same product by the same organism. Reaction sequence B initiated by benzylic oxidation appeared to be of minor

Figure 14. Metabolic routes of Deuterium labelled p-Diethylbenzenes

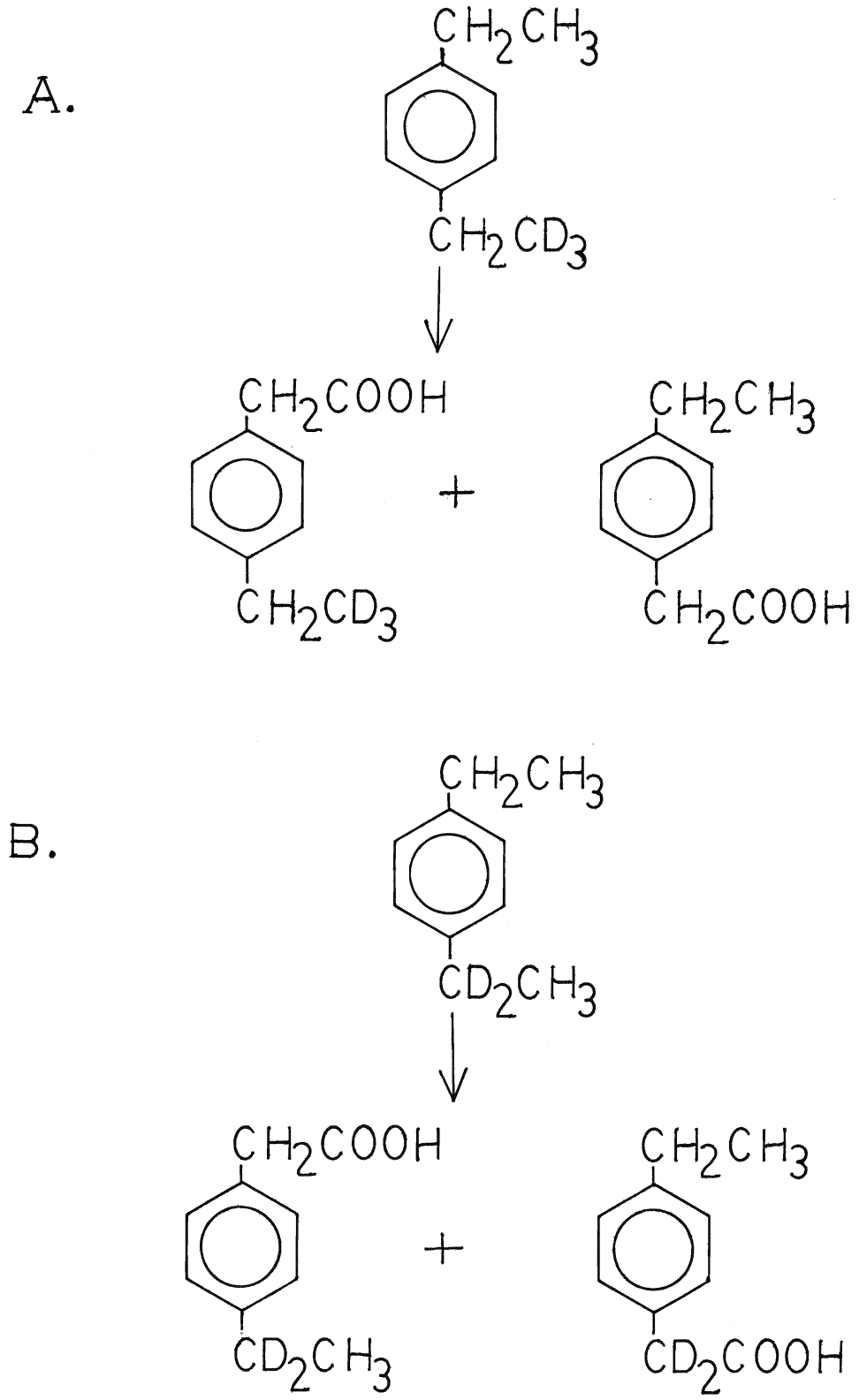
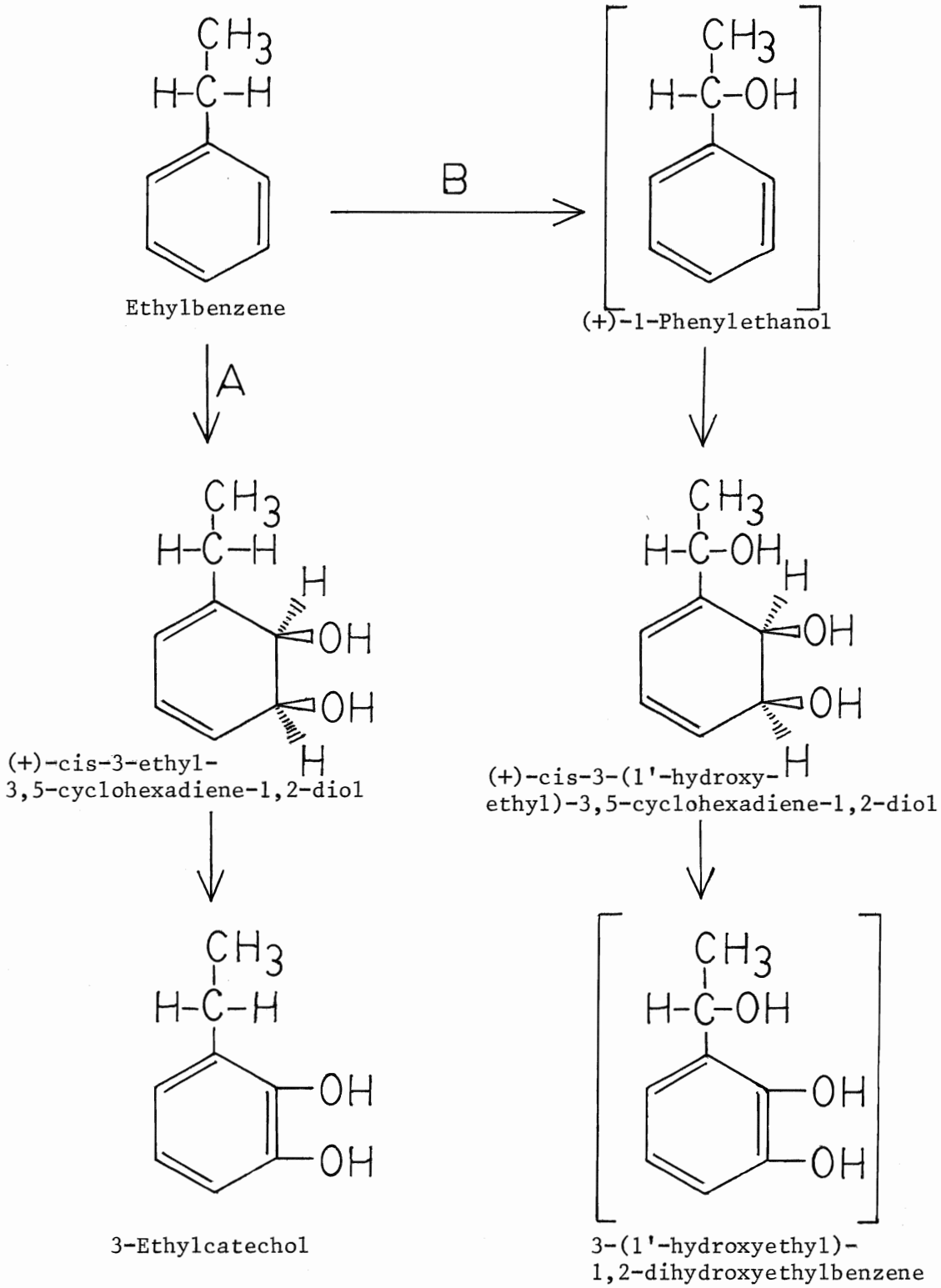


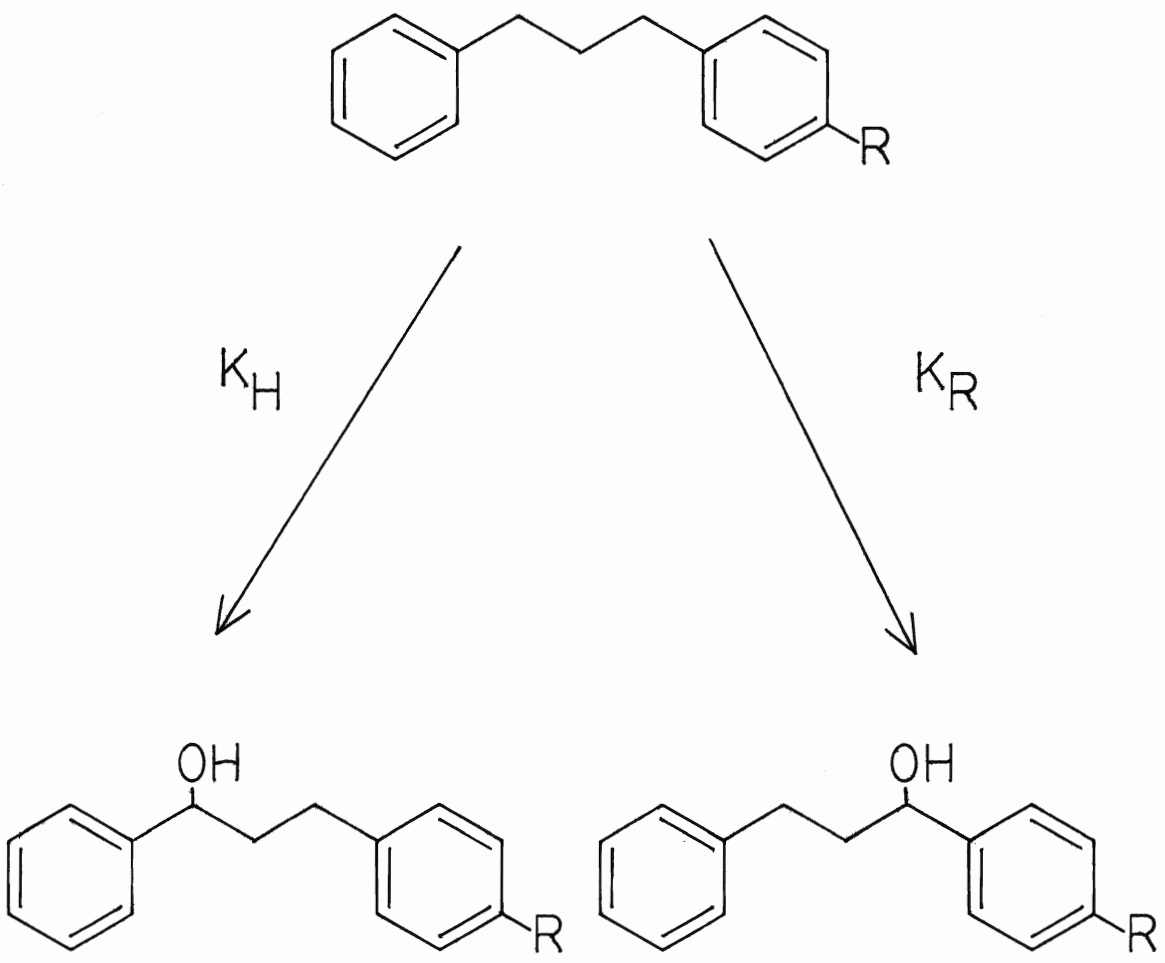
Figure 15. Reaction sequences utilized by P. putida to oxidize Ethylbenzene



importance to the organism. All the compounds except those in brackets were isolated. The authors, however, proposed their formation based on the isolation of the other componds.

Aliphatic hydroxylation reactions are generally considered to proceed *via* electrophilic reaction mechanisms.[95] Thus one would expect substituent electronic effects upon the rate of aromatic side-chain oxidations at the α position. However, studies have shown that K_m and V_{max} of such hydroxylation reactions showed little or no dependence upon electronic differences in the substituents. The rate of hydroxylation by cytochrome P-450 is probably relatively fast compared with other steps in the oxidation process. Thus substituents may significantly alter the rate of oxidation without this being reflected in the rate of product formation. These problems have been overcome by the use of substituted 1,3-diphenylpropanes to study substituent effects upon hydroxylation at the benzylic carbon atom.[96] Such compounds have the advantage that there is an intramolecular competition for hydroxylation at the two benzylic positions (Fig. 16). The ratio of the rates of hydroxylation at the benzylic positions k_H:k_R may, therefore, be determined by measuring the ratio of two products in the reaction mixture without reference to the absolute rate of hydroxylation. Problems arising from steric hindrance may be minimized by selection of appropriate substitutents. The compounds used were R = H, F, CH_3 and CF_3. With fluorine and trifluoromethyl substituent, 70% and 95% respectively,

Figure 16. Hydroxylation of substituted 1,3-Diphenylpropanes



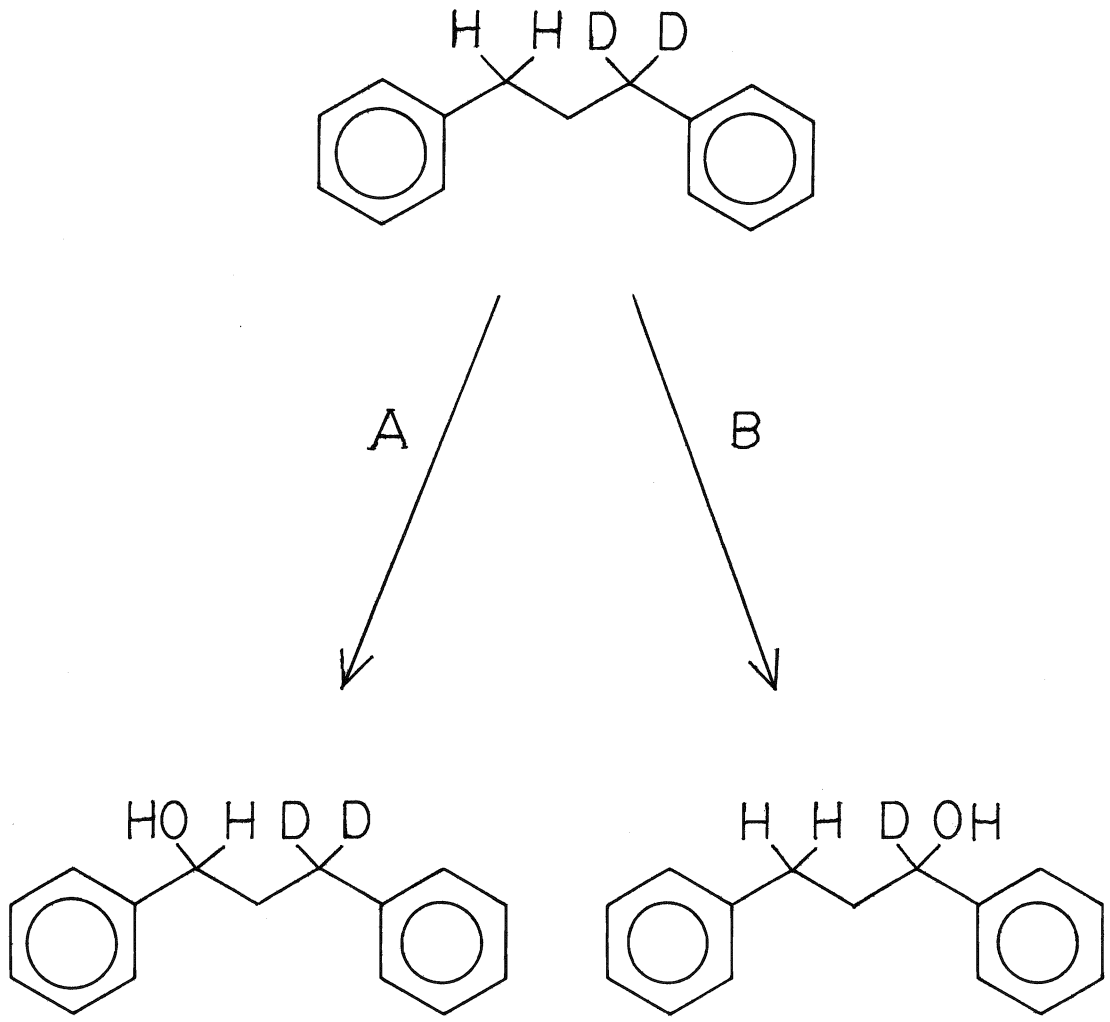
R = H
R = F
R = CH_3
R = CF_3

of the product was hydroxylated at the benzylic carbon of the nonsubstituted benzene ring. Methyl substitution did not result in selective hydroxylation of either benzylic position. When CF_3 is compared with CH_3 and F is compared with H (comparisons which minimize the effects of steric hindrance), it is apparent that the position of hydroxylation is oriented away from electron withdrawing groups. This demonstrates that aromatic side-chain hydroxylation occurs by an electrophilic mechanism.

The same authors[97] have studied the isotope effect of hydroxylation reactions using 1,3-diphenyl-[1,1-d_2]-propane which is deuterated at one of the benzylic positions (Fig. 16). This molecule has the advantage that there is an intramolecular competition for hydroxylation at the two benzylic positions. Thus the isotope effect $k_H:k_D$ may be measured simply by determination of the ratio of the fully deuterated product to that in which one deuterium atom has been replaced by a hydroxyl group (Fig. 17). The results from incubation with rat liver microsomes gave a value of $k_H/k_D = 11$, which is much greater than previously reported values for benzylic hydroxylations by microsomal monoxygenases. On the basis of these results, the authors suggested a radical abstraction-recombination as a possible mechanism for aliphatic hydroxylation reactions.

In a recent paper, McIntire *et al*[98] have described enzymic hydroxylation of p-ethylphenol by highly purified p-cresolmethyl

Figure 17. Microsomal hydroxylation reaction of 1,3-Diphenyl-[1,1-d_2]propane



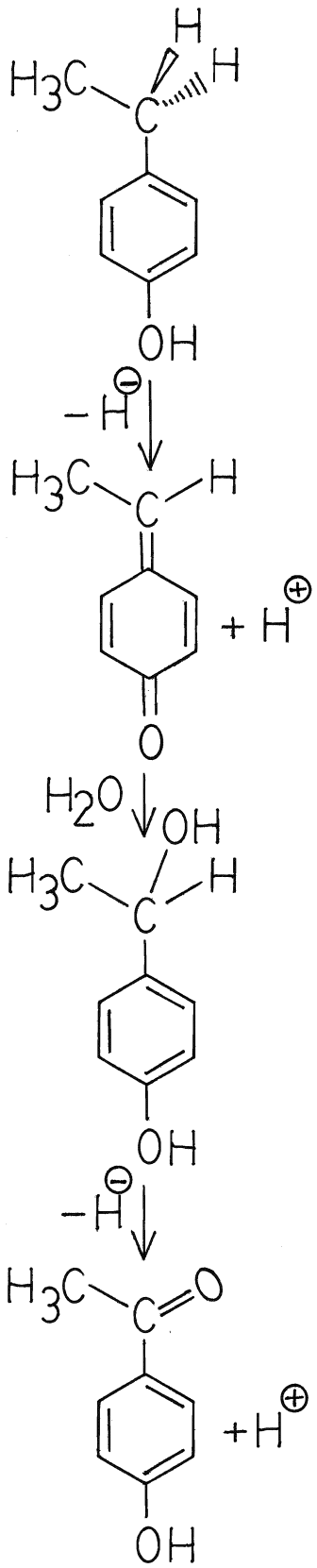
hydroxylase and Pseudomonas putida which gave optically active 1-(p-hydroxyphenyl)ethanol. The products were transformed into the phenolic methyl ethers and shown to contain enantiomeric excesses of 31.2% and 39.0%, respectively, of the (S)-(-)-isomer. The stereochemistry of the reaction catalysed by p-cresolmethyl hydroxylase is discussed in terms of three distinct and potentially stereospecific steps occurring at the active site of the enzyme (Fig. 18), but since the enzyme is not a monooxygenase, the relevance of this finding to the mode of action of cytochrome P-450 dependent monooxygenases is unclear and, therefore, not being discussed here in detail. The stereoselectivity of product formation by P. putida was not commented upon.

In addition to ethylbenzene, propyl and butyl benzenes have also been reported to be metabolized by a variety of bacterial strains isolated with isopropyl benzene as carabon source.[99,100]

Apart from the formation of 1-phenylethanol, the oxidation of ethylbenzene by rat liver microsomes also produced 4-hydroxyethylbenzene and 2-hydroxyethylbenzene.[101] Now, it is apparent from the literature that the ethylbenzene molecule possesses several different positions that are susceptible to oxidation. The site of the initial reaction varies with the source and specificity of the enzyme system involved.

Considerable information is available on oxidation of ethylbenzene by mammalian hepatic microsomes but at this time there have been no

Figure 18. Reactions catalysed by p-Cresolmethyl hydroxylase



* Hypothetical intermediate

detailed reports on the metabolism of ethylbenzene by cytochrome P-450 dependent monoxygenase present in a fungi.

Recent studies[102] undertaken in our laboratory have demonstrated that a series of para-substituted alkyl aryl sulphides have been transformed stereoselectively to the corresponding sulphoxides by incubation with the fungus Mortierella isabellina . The enantiomeric purity of the products was dependent on the electronic nature of the para substituent. Since the sulphur oxidation of alkyl aryl sulphides and benzylic hydroxylation at saturated carbon are believed to have electrophilic character,[95] although their connection has yet to be established, if the same monooxygenase system is carrying out both the oxidations, then it may be proposed that ethylbenzene and its para substituted analogs can be transformed to optically active alcohols and stereoselectivity will be susceptible to electron withdrawing or donating nature of para substituents.

The present study describes an analysis of metabolites formed from alkylbenzenes by Mortierella isabellina NRRL 1757. In addition to the product formation, steric and electronic effects of sbustitution at the para position of the aromatic ring on the degree of enantiomeric enrichment of the resultant chiral alcohols are discussed. To our knowledge, this seems to be the first study which represents the ethylbenzene transformation to optically active alcohol by fungal cytochrome P-450 dependent enzyme.

PART THREE

Microbial Reduction of Ketones

Primary studies[85] on the *in vivo* reduction of acetophenone to only one stereoisomer, i.e., (-)-1-phenylethanol have established that an enzyme which mediates the stereospecific reduction of acetophenone is also present along with the enzyme which catalyses the nonspecific hydroxylation of ethylbenzene to racemic 1-phenylethanol in rabbit body.

Culp and McMahon[103] have described the reduction of acetophenone and its para substituted analogs by an enzyme, aromatic aldehyde ketone reductase. A-K reductase, however, does not catalyze the reverse reaction under the experimental conditions used. The effect of ring substitution on the rate of reduction was of interest. Electron-withdarawing groups greatly enhance the rate, while the electron donating group enforces a reduced rate.

A-K reductases[104] are usually NADPH dependent enzymes found in the cytoplasmic fraction of the cell and have very broad substrate specificity.

The reduction of a series of aliphatic and aromatic ketones has been shown to be catalysed by enzymes in rat liver which differ in cofactor requirements from LAD.[105] The cytosol is the predominant site for reductions. Substrates in which at least one aromatic ring is adjacent to the carbonyl group are reduced to alcohols in NADPH dependent reactions. Aliphatic ketones are reduced equally well in the presence of NADH and NADPH. Acetophenone reductase was not enhanced by

pretreatment of rats with phenobarbital. The enzyme responsible for the reduction of aromatic ketones was thought to be probably identical with that described by Culp and McMahon.[85]

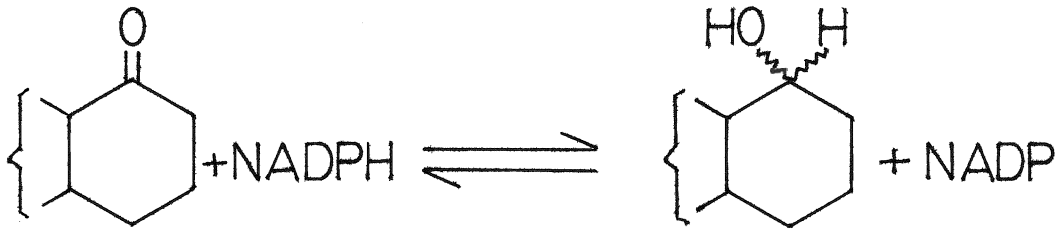
Reductions of ketones[106] are often catalysed by the oxidoreductases that oxidise alcohols. Reveresibility can be demonstrated by using the appropriate substrate and cofactor. Cyclic ketones are reduced by an enzyme present in human liver. The enzyme activity is located in the cytosol, and is not found in the microsomal fraction; it utilizes NADPH more effectively than NADH. The reaction occurs in the presence and absence of oxygen. It is readily reversible, alcohol is oxidized to ketone in the presence of crude enzyme preparation and NADP. The pH optimum for the reverse reaction is higher than that for the forward reaction, but it is not known whether the same enzyme catalyses both (Fig. 19).

Cervinka and Hub[107] have shown the stereoselective reduction of p-nitro and p-methoxy acetophenone using yeast, Saccharomyces cerevisiae. In both the cases, an excess of laevorotary alcohols was obtained; the two alcohols were assigned S configurations.

A detailed discussion of these reductions is beyond the scope of this thesis, however, enzyme catalyzed reduction of ketones, producing chiral alcohols has been extensively documented.[108]

This study deals with the stereoselective reduction of carbonyl group present in acetophenone, α,α,α-trifluoroacetophenone and p-ethylacetophenone to the corresponding chiral alcohols, using

Figure 19. Microbial reduction of Ketones



Mortierella isabellina NRRL 1757. This metabolism may be of considerable importance to synthetic organic chemist as it provides a convenient pathway to produce chiral alcohols in high optical purity and good chemical yield which may otherwise be difficult to obtain.

PART FOUR

Cytochrome P-450 : A Unified View

Cytochrome P-450 is the collective name for a distinct group of protoheme containing proteins which show a characteristic absorption band at around 450 nm in the carbon monoxide difference spectrum of dithionite reduced samples.

Cytochrome P-450 was first described in mammalian liver microsomes and was originally thought to be occurring only in restricted biological systems.[59] However, subsequent studies have revealed that heme proteins with similar spectral properties are distributed very widely in nature, they can be detected in almost all forms of life.[59,95] In mammals, cytochrome P-450's are found at varying concentration in microsomes (endoplasmic reticulum) of the liver, kidney, small intestine, lung, adrenal cortex, skin, testes, placenta and several other tissues. The enzyme system that has received most of the attention is that of mitochondria and liver microsomes. Cytochrome P-450's have also been detected in bacteria, yeasts and fungi. These enzymes exist in both soluble and membrane bound forms.

These enzymes, which incorporate one molecule of molecular oxygen into the substrate, are classified monooxygenases and carry out oxidations with the stoichiometry shown below.[109]

$$R\text{-}H + NAD(P)H + H^{+} + O_2 \longrightarrow R\text{-}OH + NAD(P)^{+} + H_2O$$

The specificity of this monooxygenase is also different than that

of most enzyme systems, which are usually specific for one group of substrates. This enzyme system is capable of catalyzing the oxidation of a wide variety of compounds. Not only is it able to react with polycyclic aromatic hydrocarbons and steroids,[95,110] but also with fatty acids, alkanes, drugs and a variety of foreign compounds.[111] It appears to be one of the most versatile catalysts available for many reactions[110,112] such as hydroxylation and epoxidation. Besides these main functions, P-450 also catalyzes[113,114] sulphoxidation, N- and O-demethylation, deamination, N-oxidation and other reactions[115] of a range of different substrate types. Because of the diversity of substrates and the variety of transformations that these enzymes execute, this family of cytochromes has attracted the attention of several groups in many fields including organic, inorganic and biochemistry, pharmacology, bacteriology, toxicology and oncology. Interest in the latter two fields has been particularly great because of the ability of these enzymes to metabolize rather inert compounds to electrophilic products that can bind irreversibly to cellular macromolecules and thus resulting to the ultimate development of toxic or tumorigenic lesions. This aspect of the cytochrome's function has been discussed in a previous section. In addition to its biological significance, this enzyme now constitutes a formidable synthetic arsenal. Cytochrome P-450 is capable of directly introducing a hydroxy group into an organic molecule by performing the direct conversion of carbon-hydrogen bond to carbon-hydroxyl with defined regio- and

stereospecificity.[95] Thus, cytochrome P-450 is also a "catalyst" which can be explored by the synthetic organic chemist to produce many compounds which are otherwise inaccessible to produce by conventional chemical means.

The reason for the wide range of substrate specificity is not quite understood, but this has been partly attributed to multiple forms of cytochrome P-450 and was supported by the observation of variable activities in animals which were treated with different P-450 inducing agents. The most common inducing agents used are phenobarbital and 3-methylcholanthrene.

Since its discovery over two decades ago, cytochrome P-450 has been an object of intensive research by both organic and biochemists, and a large amount of data has been compiled.[59]

In attempting to understand how the enzyme performs the reactions, an accurate description of its structure and how the active site works is required.

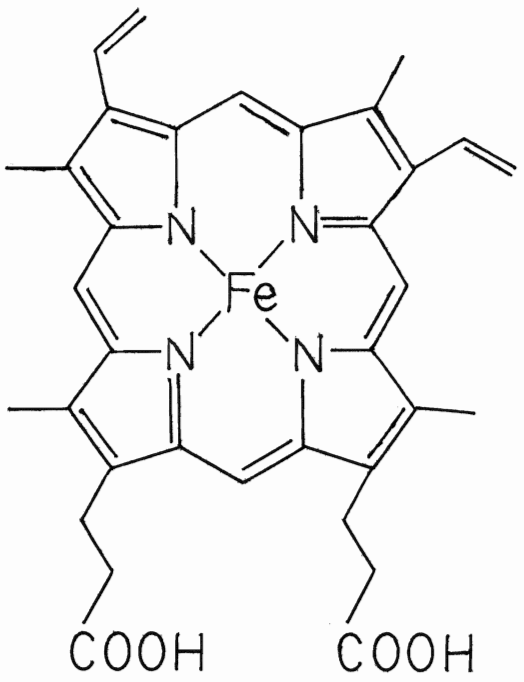
The problem with working with a cytochrome P-450 system is that it is difficult to isolate and purify a soluble form of the enzyme. This is why the enzyme preparation from camphor grown Pseudomonas putida is important; it is soluble and is thus most precisely defined cytochorme P-450 and has been obtained in a crystaline form.[116] Much of the mechanistic work on this class of enzymes has been performed using soluble enzymes obtained from camphor hydroxylating enzyme of P. putida [59,117] or on enzymes of mammalian origin, but the available

evidence suggests that all cytochrome P-450 monooxygenases function by a similar mechanism.[59]

The actual structure of cytochrome P-450 can be divided into two parts: a protein body, the "apoenzyme" and an iron heme in the form of iron protoporphyrin protosthetic group, the "cofactor". A protoheme group containing iron(III) in the resting state of the enzyme, is coordinated to a macrocyclic tetrapyrrole ring (Fig. 20).[95] The pyrrole nitrogens of this ring make up four ligands to the iron in equatorial positions, which leaves the two axial positions to be filled by other ligands. These two ligands are provided by the protein; one of these is a cysteine sulphide ion, while the other, which is displaced by oxygen during the catalytic cycle, is currently unidentified but may be imidazole nitrogen of a histidine residue. This is how the catalytically active heme cofactor is bound to the apoenzyme.

The role of apoenzyme and cofactors may be distinguished as follows: the apoenzyme is responsible for the binding of the substrate; and cofactors are responsible for the binding of oxygen, its activation and delivery to the substrate of the oxidizing species. The apoenzyme has at least three binding sites for the substrates so that the substrate can be bound in a stereospecific manner and this is how it controls the substrate regio- and stereo-specificity of hydroxylation reaction. There are different types of cytochrome P-450 which have different protein portions (i.e., the amino acid sequence) of the enzyme, and thus differ in bonding pattern of substrate,[118,119] and

Figure 20: Protoheme group of Cytochrome P-450



this variation is largely responsible for wide range of the substrate specificities and product formation.[95]

The multiple forms of cytochrome P-450 function with different substrate, regio- and stereo-specificities, but the available data from mammalian systems suggest that all are dependent on the same cofactors.[120,121] The close similarity between reactions performed by mammalian and fungal systems as discussed by Smith and Rosazza[122] suggests that a parallel state of affairs may also exist between the two at the enzymic level. The binding and the activation of oxygen, as well as its transfer to the substrate is described in the catalytic cycle of cytochrome P-450 illustrated in Figure 21. The cycle has been mainly deduced from studies of the soluble cytochrome $P\text{-}450_{cam}$. The first step, substrate binding, is necessary before the enzyme can bind oxygen or accept any reducing equivalents. The substrate does not bind directly to the heme unit, but is presumably bound by the apoprotein in close proximity to the cofactor.[95] When cytochrome P-450 is in its resting state, i.e., not bound to substrate or oxygen, the enzyme is in the iron(III) state in a low spin form.[123] In this case, the 5d electrons distribute themselves in the three low lying dxy, dyz and dxz orbitals. Since there are no electrons in the Eg orbitals, the $dx^2\text{-}y^2$ and dz^2 orbitals are empty and this spin state is more compact. Therefore the six ligands around the iron atom are arranged into an octahedral complex (Fig. 22). Substrate binding by the cytochrome P-450 can promote a change of the iron(III) from low spin to

Figure 21. The catalytic cycle of Cytochrome P-450 dependent monooxygenases. (S = substrate)

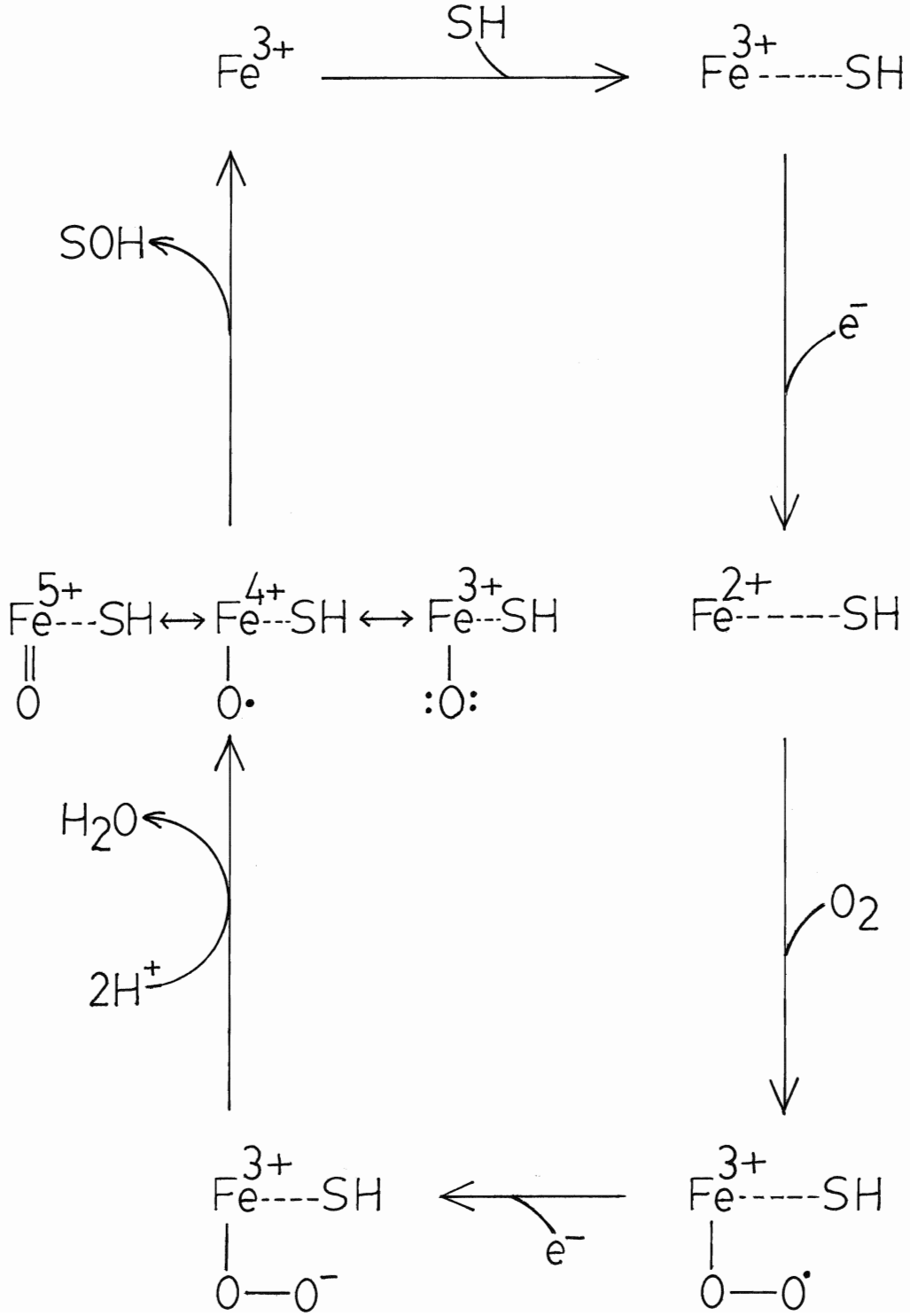
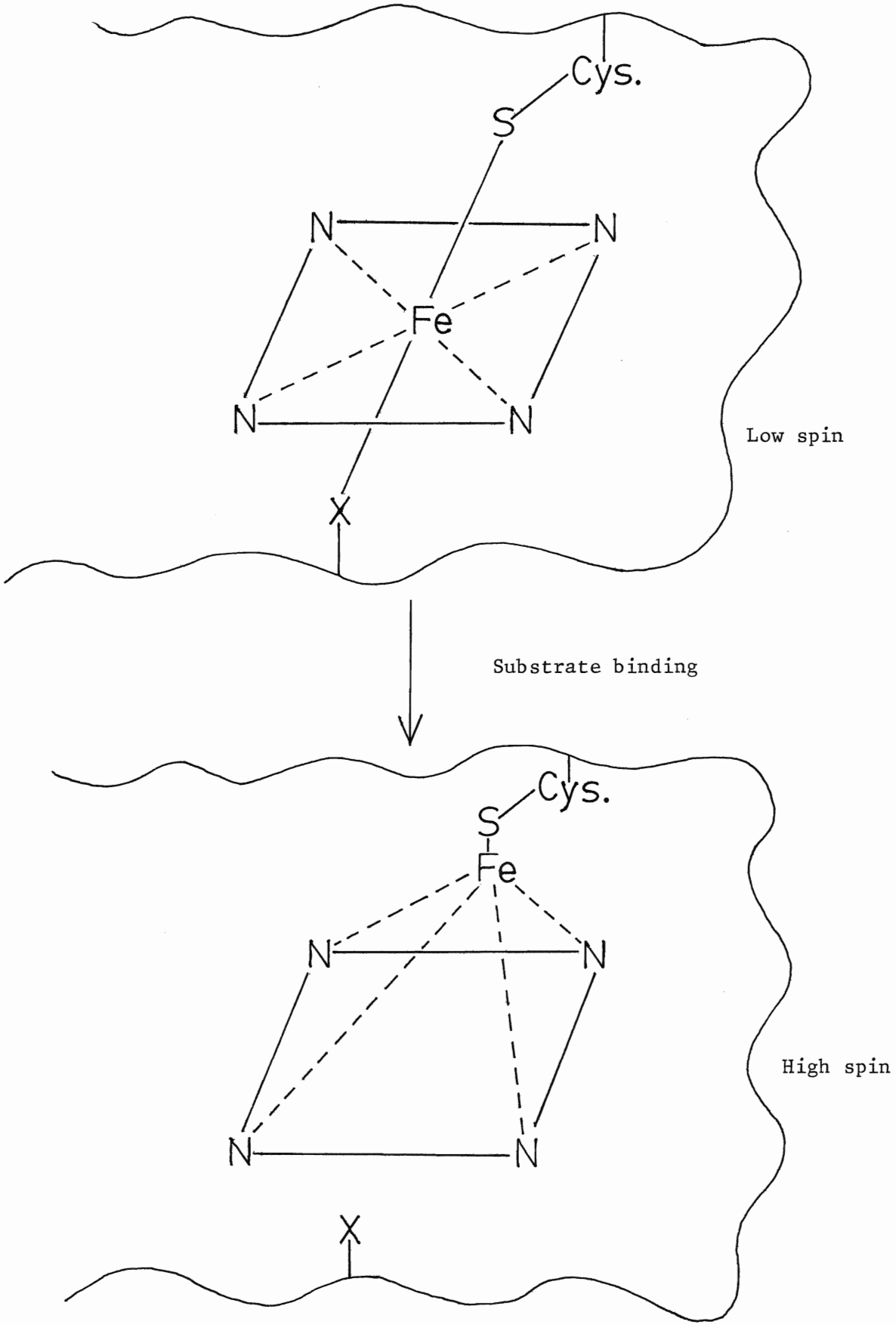


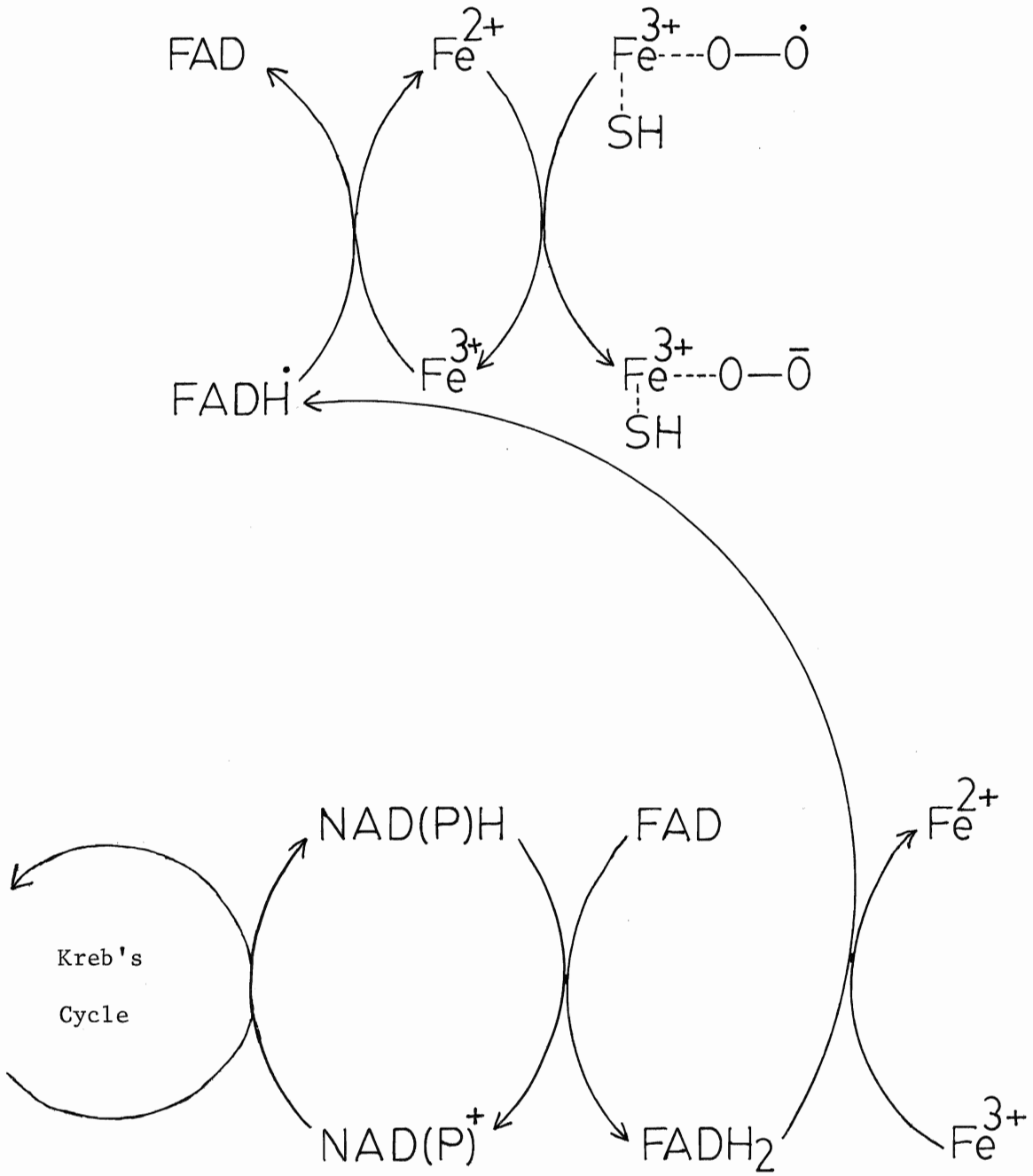
Figure 22: Spin transition in Cytochrome P-450



a high spin electronic configuration. In the high spin state, one electron on iron enters the dx^2-y^2 orbital. The four lobes of this orbital occupy the same space as that occupied by the lone pairs of electrons on the porphyrin nitrogens. Therefore, when an electron enters this orbital, the actual diameter of the iron atom is increased. It then no longer is capable of resting in the cavity of the planar porphyrin ring but is above the plane.[124] A change in coordination number from six to five is believed to accompany this transformation, and as a result the interaction with the sixth unidentified ligand is broken.

In the next step of the catalytic cycle, the cytochrome substrate complex undergoes a one electron reduction. This electron is ultimately derived from the oxidation of Kreb's cycle intermediates and is transferred to the cytochrome P-450 *via* a complex array of cofactors (Fig. 23). The first cofactor, NAD(P)H, is provided by the oxidation of Kreb's cycle acids. It passes its reducing equivalent to a flavin cofactor, FAD. The product of this initial transfer, $FADH_2$, passes only one electron to an iron-sulphur protein (ferredoxin). This leaves the flavin cofactor at the level of $FADH^{\bullet}$. Finally, the iron sulphur centers of ferredoxin carry out the actual reduction of the iron center of cytochrome P-450. This sequence of cofactors is applicable for electron transfer in the case of camphor grown *P. putida*, while

Figure 23. Reduction of Cytochrome P-450



in hepatic microsomal systems the iron-sulphur protein is replaced by cytochrome b_5.[125] In either case, these cofactors use the reducing equivalents generated by the Kreb's cycle to reduce the prosthetic group of cytochrome P-450. The reduced heme iron is then ready to bind and activate oxygen.

The activation of oxygen is initiated by its binding to reduced cytochrome P-450. Because the oxygen is a good electron acceptor, this step is quite rapid.

The binding of molecular oxygen to the porphyrin iron results in the formation of iron-oxy complex [SH-Fe(III)-$\dot{O}_2$]. The system is now capable of receiving a second electron which is provided by FADH$^{\bullet}$ bringing the flavin cofactor back to the fully oxidized state, FAD (Fig. 23). The product of this second reduction is an iron-peroxo complex, [SH-Fe(III)-O-O^{-}]. At this stage, the enzyme complex becomes the active species which dissociates readily to product and water and regenerates the iron in free state. During this process, one atom of molecular oxygen is given to a proton to produce the water molecule. After the addition of the second electron, the mechanism becomes unclear because of this rapid dissociation. At the present time there is no suitable technique for trapping the active oxygen species in cytochrome P-450. Most of the data obtained regarding this species has resulted from the studies of oxidation of organic substrates and from chemical models of this enzyme.

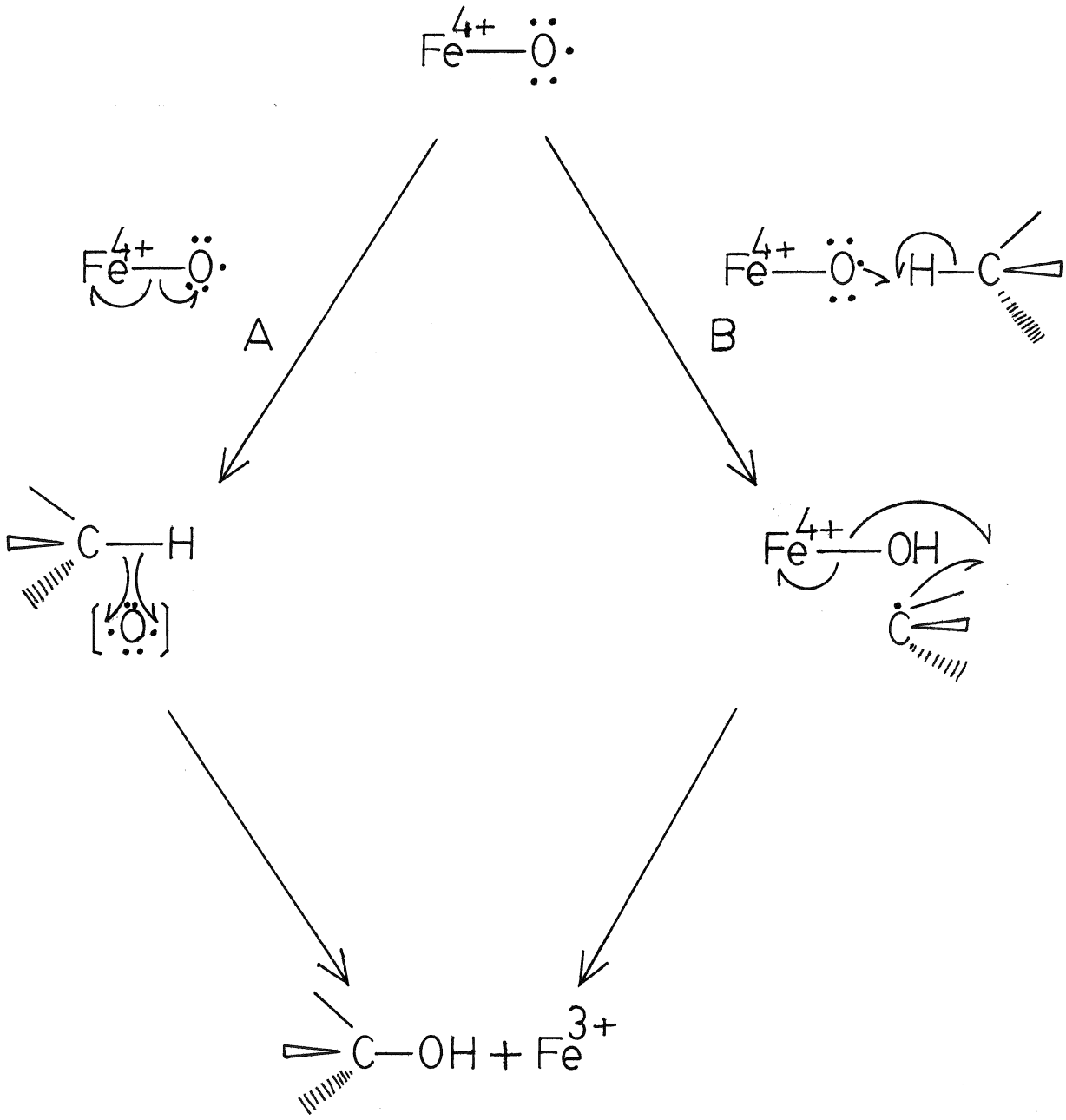
Although cytochrome P-450 dependent monooxygenases from different sources show differences in electron-transport cofactor requirements,

the available data suggest that overall features of the catalytic cycle of these enzymes are largely independent of the source.[59]

It has always been assumed that no specific activation of the C-H bond in the substrate is provided by the enzyme. The position of hydroxylation is controlled by the geometrical nature of the active site. However, not all C-H bonds of a substrate are equally reactive towards hydroxylation.[95]

The nature of the enzymically activated oxidising species is now clear, and factors which can control the binding are understood. It is now possible to examine the actual mechanism of hydroxylation. This aspect of the hydroxylation process is not yet fully understood. However, in recent years the route by which the activated oxygen complex of figure 21 reacts with a C-H bond has been the subject of extensive investigations. Two favoured mechanistic pathways have been proposed (Fig. 24), a concerted insertion of electrophilic oxygen ("Oxene") into a C-H bond of substrate (Fig. 24, path A)[126] and a radical abstraction-recombination mechanism (Fig. 24, path B).[127] Evidences for both are available but which one of these mechanisms is correct has still not been resolved. This lack of agreement is well illustrated by a recent study involving the oxidation of ethanol by cytochrome P-450, which supports both the mechanisms.[128] The authors proposed that both pathways must be correct but which one will be dominant in any particular case would be determined by the environment of the enzyme. For example, in the presence of hydroxyl radical scavenger molecules (e.g., DMSO, benzoate), only the concerted mechanism would work. Therefore, in special circumstances both pathways

Figure 24. Possible routes for hydroxylation at saturated carbon



may operate.

In most cases experimental data favours only one mechanism for cytochrome P-450 hydroxylation. The oxene mechanism of hydroxylation (Fig. 24, path A) has been accepted as being the most consistent with the results obtained in various studies. First, cytochrome P-450 catalysed hydroxylation proceeds with retention of configuration at carbon center.[129] If substrate bonds were broken and free radicals formed, some type of rearrangement or racemization could occur. Secondly, a closely related reaction, epoxidation, is also carried out by cytochrome P-450 dependent monooxygenases[130] utilizing the same active and binding sites.[131] Thus both hydroxylation and epoxidation must proceed by essentially the same pathway. The epoxidation of alkanes by peroxyacid has been shown to occur via a concerted insertion mechanism.[132] Therefore, if the enzymic process is identical to the chemical pathway, the concerted mechanism must be correct.

Further evidence for the concerted mechanism came from the studies with aromatic systems. Hydroxylation of these compounds proceeds with the intramolecular migration of the substituent from the position where the hydroxy group is induced, to either adjacent ortho positions. This phenomenon is called "NIH shift" (Fig. 2).[14] The observation that the non-enzymatic isomerization of arene oxides showed rearrangements compatible with the migrations that take place during NIH shift led to the proposal that an intermediate arene oxide might be involved in these

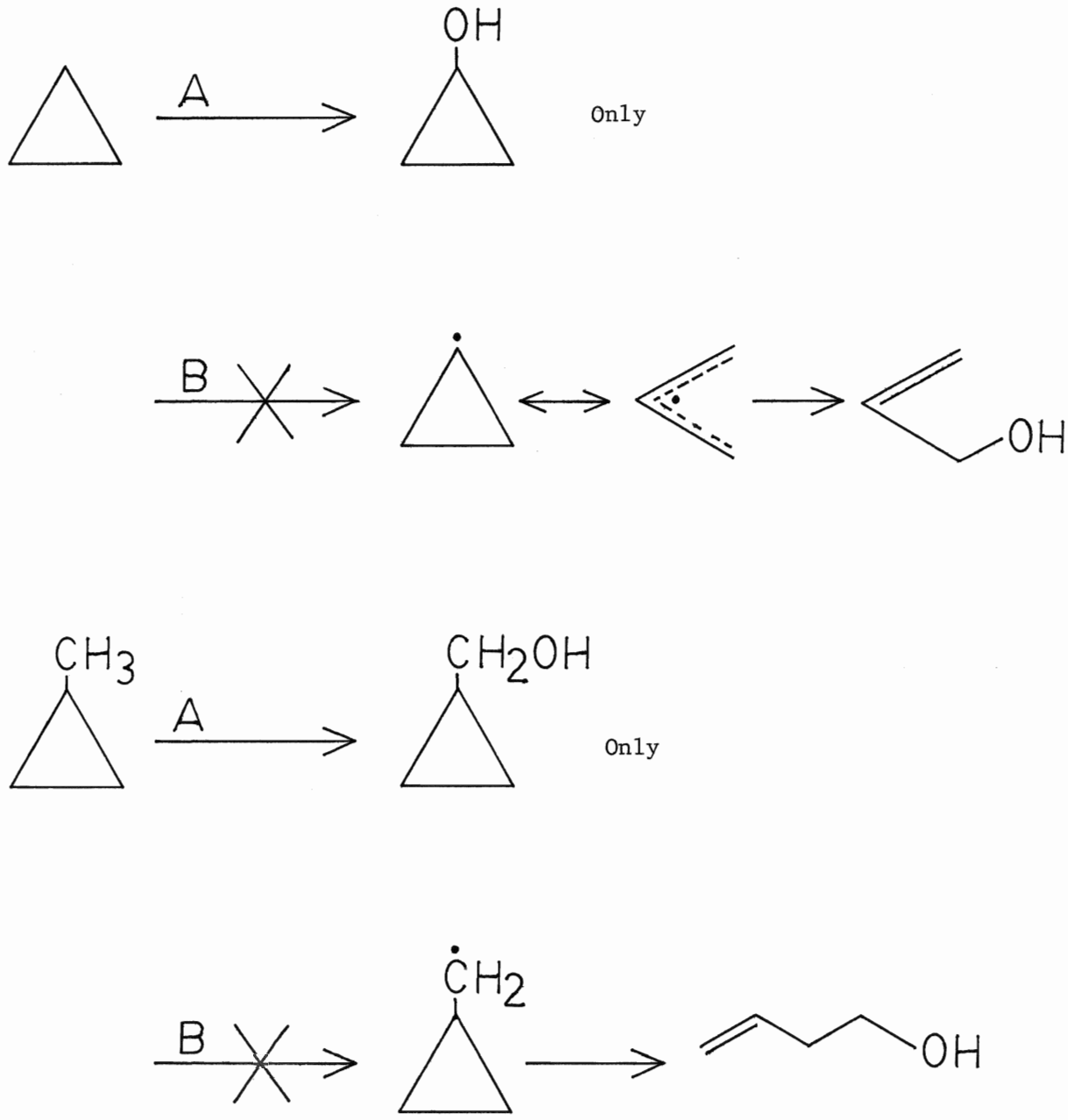
reactions.[133] Jerina *et al*[134,135] reported the isolation of 1,2-napthalene oxide from the incubation of napthalene with rat liver microsomes. The presence of this arene oxide intermediate was explained by an addition of an oxene to a carbon-carbon double bond.

Dalton and co-workers[136] examined the cytochrome P-450 catalyzed hydroxylation of cyclopropane and methylcyclopropane using *Methylococcus capsulatus* as the enzyme source. No rearrangement products were detected, supporting the electrophilic insertion mechanism. Cyclopropanol and cyclopropyl methanol were observed as the only metabolic products from cyclopropane and methylcyclopropane, respectively. If any electron deficient species such as a cyclopropyl carbocation or a cyclopropylmethyl radical were formed, oxidation could have led to the formation of allyl alcohol or but-3-en-1-ol. These products were not observed, indicating that free charged or radical intermediates were not involved (Fig. 25).

All these results, plus the fact that cytochrome P-450 catalysed hydroxylation proceeds with a very low intermolecular isotope effect (generally $k_H/k_D < 2$)[89] have been interpreted as consistent with an insertion mechanism (Fig. 24, path A).

However extensive investigations of hydroxylation mechanism also lend credence to the nonconcerted radical abstraction pathway B of Fig. 24.

Figure 25. Hydroxylation of Cyclopropane and Methylcyclopropane by M. capsulatus



The first positive indication that hydroxylation may prodeed *via* radical intermediates came from a study of the metabolism of the insecticide Dieldrin in mammals.[137] The formation of the bridged metabolite has been explained by the mechanism which involves a transannular reaction of the radical intermediate (Fig. 26).

Further evidence for radical intermediates has been provided by experiments performed by Groves and coworkers.[138] These studies showed that the hydroxylation of norbornane by a liver cytochrome P-450 system affords *exo* and *endo*-2-norborneol (Fig. 27, A and B), in a ratio of 3.4:1. The ratio of these products dropped to 0.76:1 when *exo*, *exo*, *exo*, *exo*-2,3,5,6-tetradeuteronorbornane was hydroxylated. Analysis of the mass spectra of the products obtained from deuterated substrate revealed that 25% of the *exo*-norborneol contained four deuterium atoms whereas 9% of the *exo*-norborneol contained three deuterium atoms. These results indicate a very large isotope effect ($k_H/k_D = 11.5 \pm 1$) and a significant amount of epimerization for the hydroxylation of norbornane by cytochrome P-450. On the basis of these results, the authors suggested a free radical mechanism initiated by hydrogen abstraction.

Since these early findings, the studies supporting the free radical mechanism have been growing rapidly.

In a recent study,[139] a mechanism involving a free radical intermediate has been proposed for the hydroxylation of camphor at C-5 giving 5-exo-camphor, by cytochrome P-450 system of camphor grown *P. putida*. It was shown that camphor analogues containing deuterium at either the 5-*exo* or 5-*endo* position yielded only

Figure 26. Rearrangement during Dieldrin metabolism

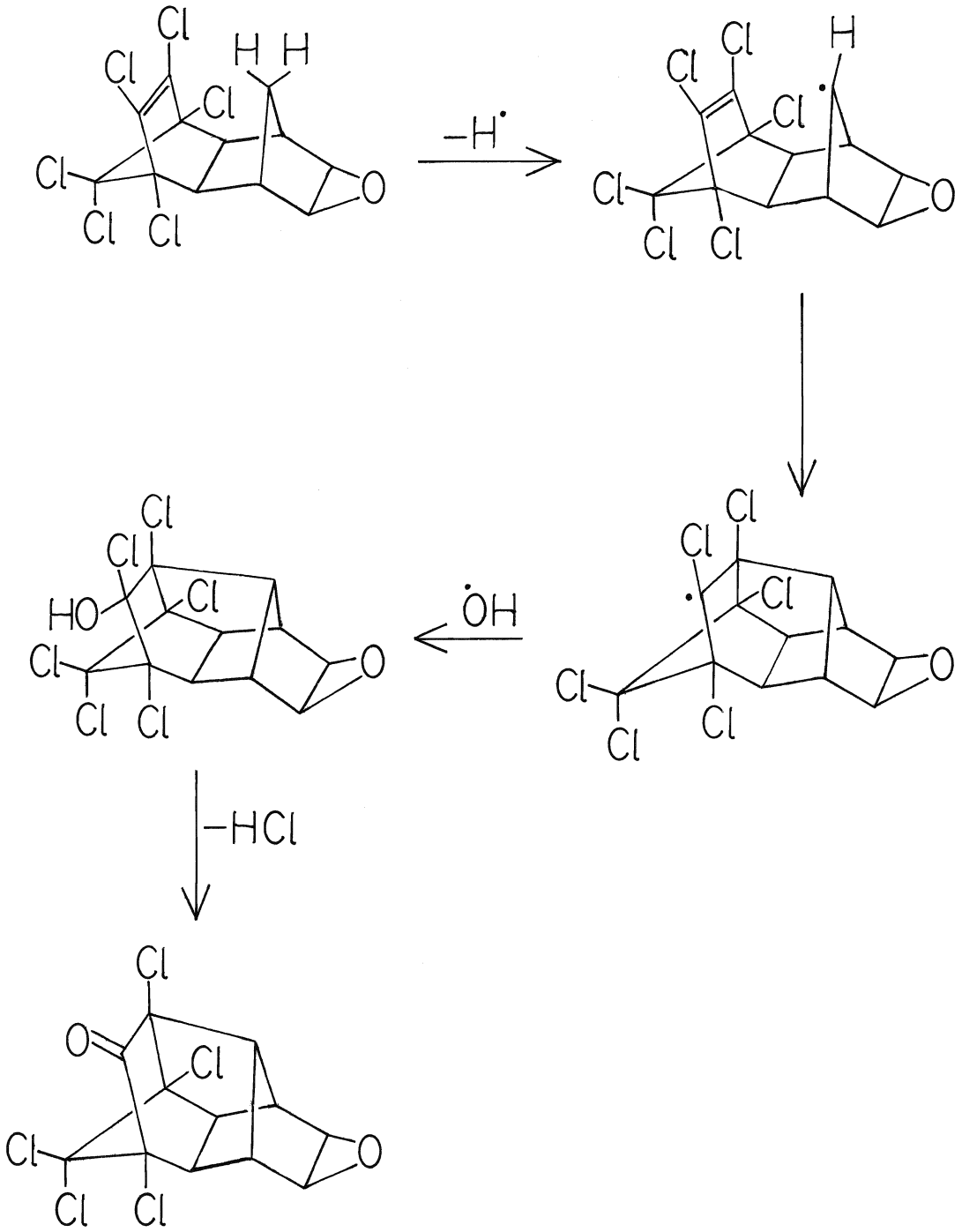
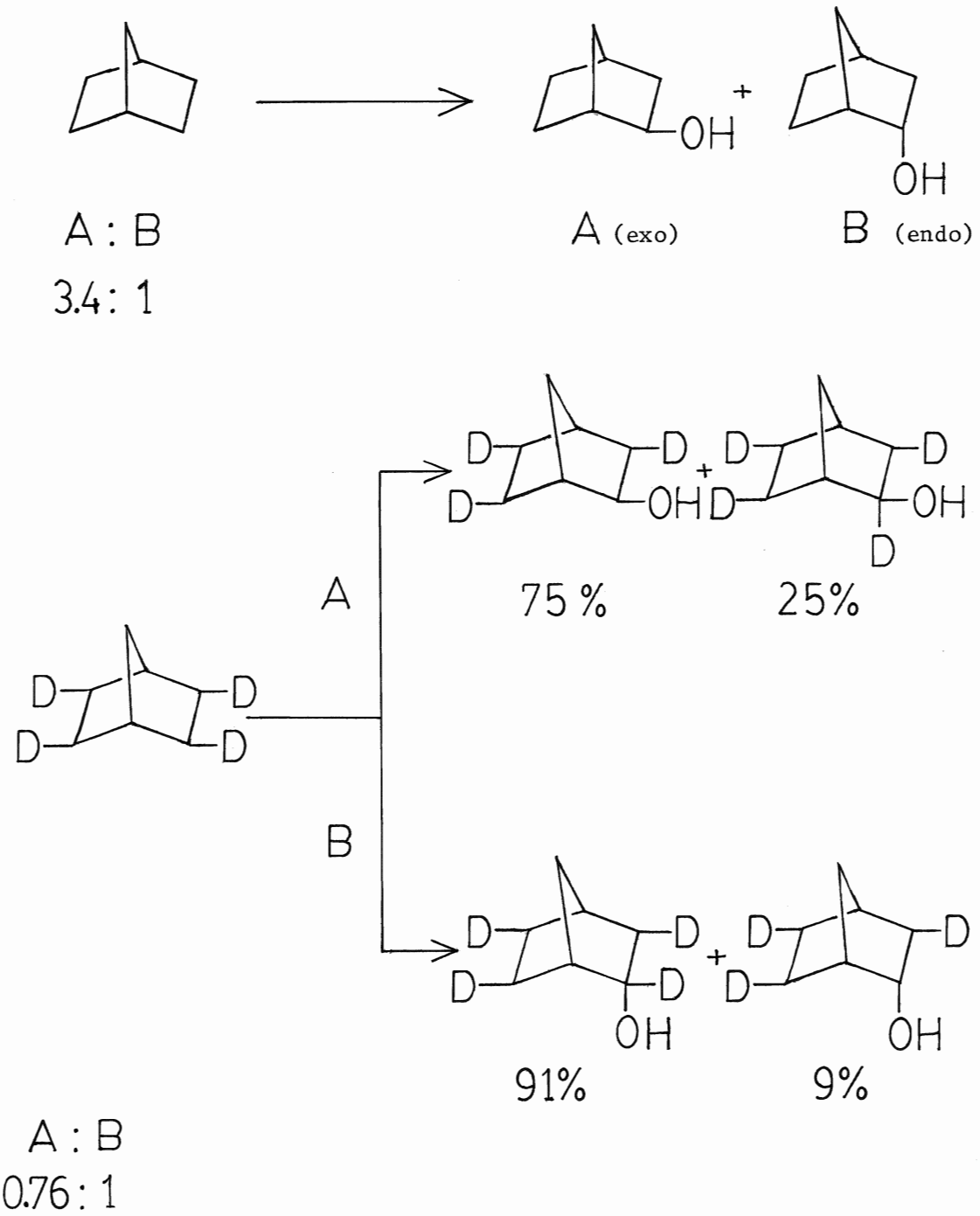


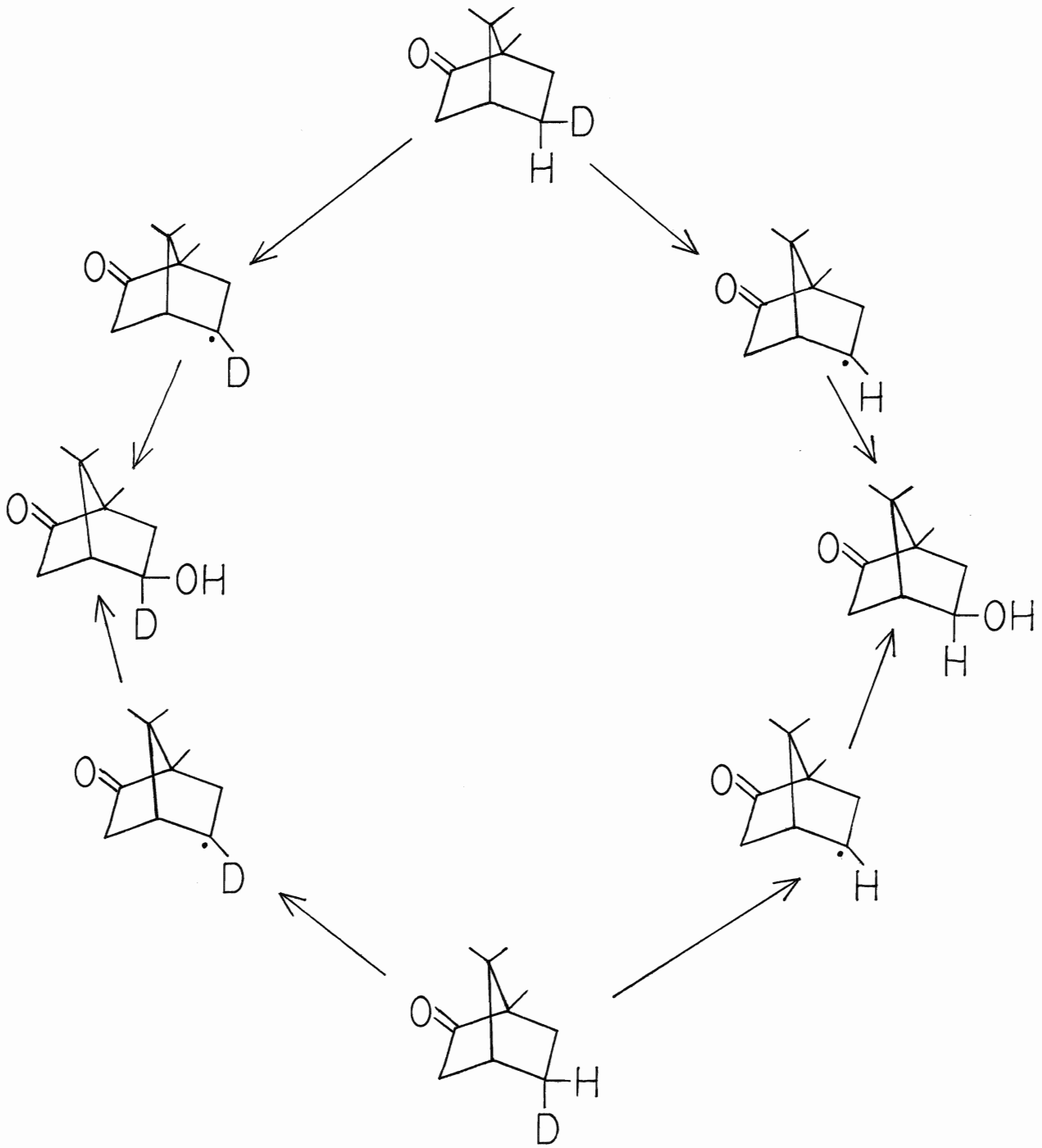
Figure 27. Hydroxylation of Norbornane by Liver Cytochrome P-450



5-exo-hydroxycamphor (Fig. 28). This reaction occurred with a substantial loss of stereochemistry at carbon 5 of camphor since the product 5-exo-hydroxycamphor retains a substantial fraction of deuterium when formed from either camphor 5-exo-d_1 or camphor-5-endo-d_1. Such a loss in stereochemistry was attributed to the partial epimerization of the substrate radical in the active site following hydrogen abstraction. A sizable intramolecular isotope effect was also observed, which supports the stepwise mechanism.

These studies are convincing in favour of a stepwise mechanism involving a free radical intermediate. However, there is some evidence which argues against this mechanism. The retention of configuration has always been used as the proof of a concerted mechanism. This observation can be explained as follows. Since the monooxygenase reactions of cytochrome P-450 are enzymic, once the substrate reacts with the enzyme it is presumably held in place by the configuration of the active site. If substrate bonds are broken during catalytic cycle of the enzyme, then any change in configuration may be hampered, and thus retention of configuration may occur. Some studies[140] have indicated that the retention of configuration observed in the enzymic reaction is not inconsistant with a stepwise mechanism. Rapid collapse of the intermediate radical formed in the enzymic reaction could also account for this observation. This means that failure to observe epimerization might be due to a relatively short living radical intermediate which reacts with the oxidising species before a complete

Figure 28. Hydroxylation of camphor by Cytochrome P-450$_{cam}$



loss of stereochemistry can take place.

The only inconsistency left is that of the intermolecular isotope effect ($k_H/k_D < 2.0$); this should be larger if a C-H bond is broken in the transition state. This aspect has also been studied quite extensively. It has been proposed that low kinetic isotope effect is a result of various factors. The rate determining step in the cytochrome P-450 cycle (Fig. 21) has been reported as the addition of the second reducing equivalent[141] and the decomposition of the P-450-substrate-oxygen complex to products.[142] It is not clear to what extent the latter steps of the catalytic cycle (Fig. 21) are kinetically distinct, and this has hampered a clear interpretation of the intermolecular isotope effects observed for hydroxylation. In most cases, such effects have been determined by product composition analysis following competitive hydroxylation of both labelled and unlabelled substrates. It is not unreasonable to assume in such a system the possible existence of isotope effects in substrate binding and other non rate-limiting steps.[143] In view of these complicating factors, the intramolecular isotope effects are now considered more important than intermolecular effects in providing the data for mechanistic studies. In cases where both inter and intramolecular isotope effects have been determined for the same substrate, the appreciable differences are clear.[144] The intramolecular isotope effects are typically large (k_H/k_D = 10-11)[97,138] and are consistent with a radical abstraction-recombination process.

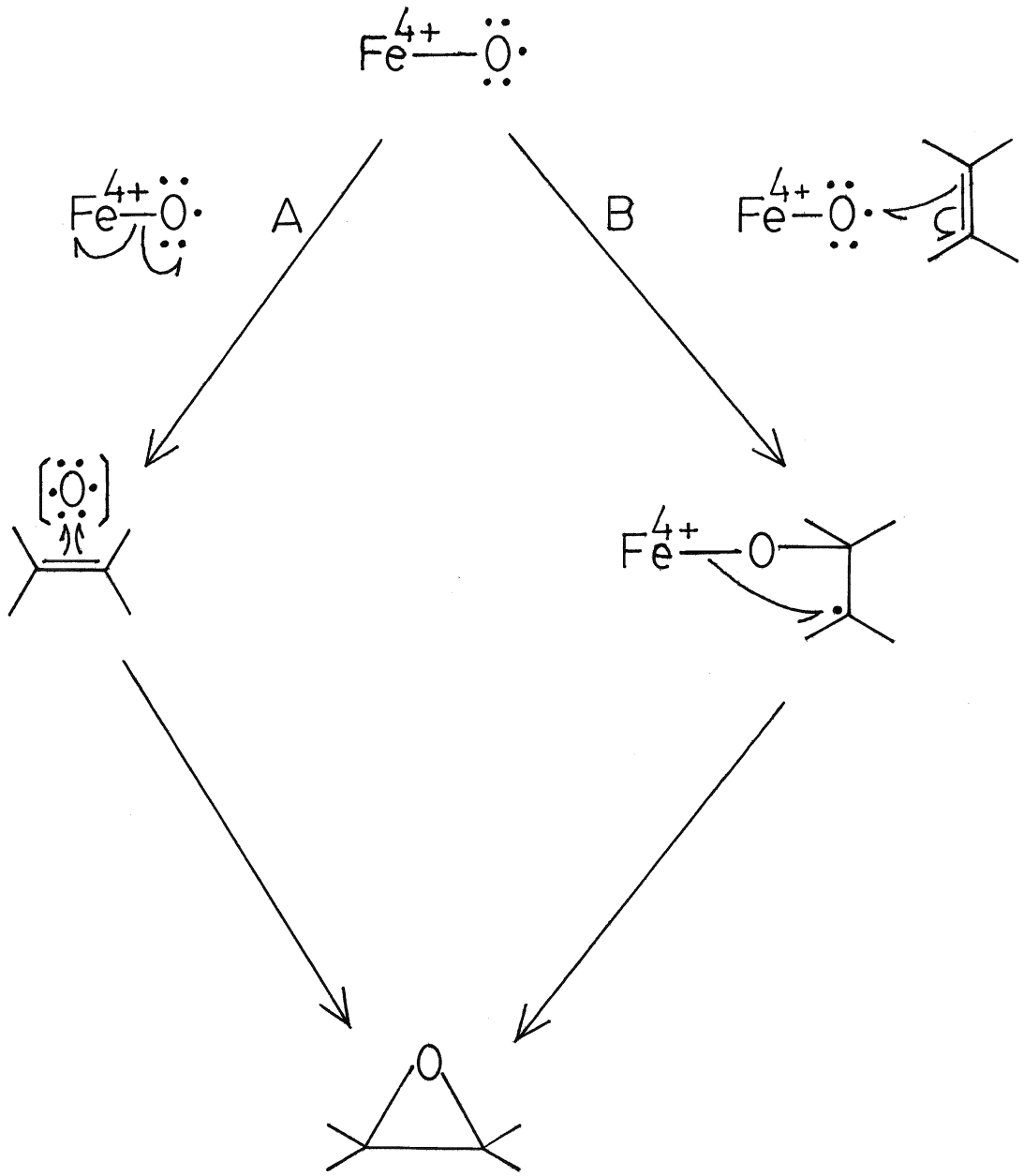
There has also been work done with cytochrome P-450 models and the results obtained were interpreted in favour of a stepwise mechanism. Work with iron porphyrin models[145-147] also suggested the formation of free radicals during the metabolic pathway. Another study[148] using iron porphyrin/iodosyl benzene catalytic system to perform hydroxylation of unactivated alkanes led to the observation of large kinetic isotope effect (k_H/k_D = 12.9 $\pm$ 1). An isotope effect of this magnitude clearly supports the radical mechanism.

The substrates discussed above are by no means the only example to confirm the operation of stepwise mechanism involving the free radical intermediate in the cytochrome P-450 catalyzed hydroxylations. Holland *et al* [110,112] have performed hydroxylation experiments with steroidal systems using *Rhizopus arrhizus* ; their results clearly support a stepwise mechanism.

A closely related reaction, also carried out by cytochrome P-450 dependent monooxygenases,[130] is the epoxidation of olefinic substrates. This reaction may proceed *via* one of the mechanisms of Figure 29, analogous to the corresponding concerted (path A) or stepwise (path B) routes of Figure 24. Evidence for both is available,[130,146] but the work of Holland and coworkers[110,112] lends credence to the stepwise (path B) rather than concerted (path A) mechanism of Figure 29.

Therefore, though the majority of the recent studies support the stepwise mechaniasms for cytochrome P-450 catalysed oxidations, these do

Figure 29. Possible routes for epoxidation at olefinic carbon



not necessarily exclude the concerted mechanism.

One final piece of information regarding cytochrome P-450, pertaining to its role in substrate sulphoxidation is that this system is capable of converting sulphides into sulphoxides and these to a certain extent into sulphones.[102,114,149] A mechanism has been proposed whose rate determining step involves an electron transfer from a divalent sulphide to the activated iron-oxygen species and a radical cation species is the involved intermediate (Fig. 30).[149,150]

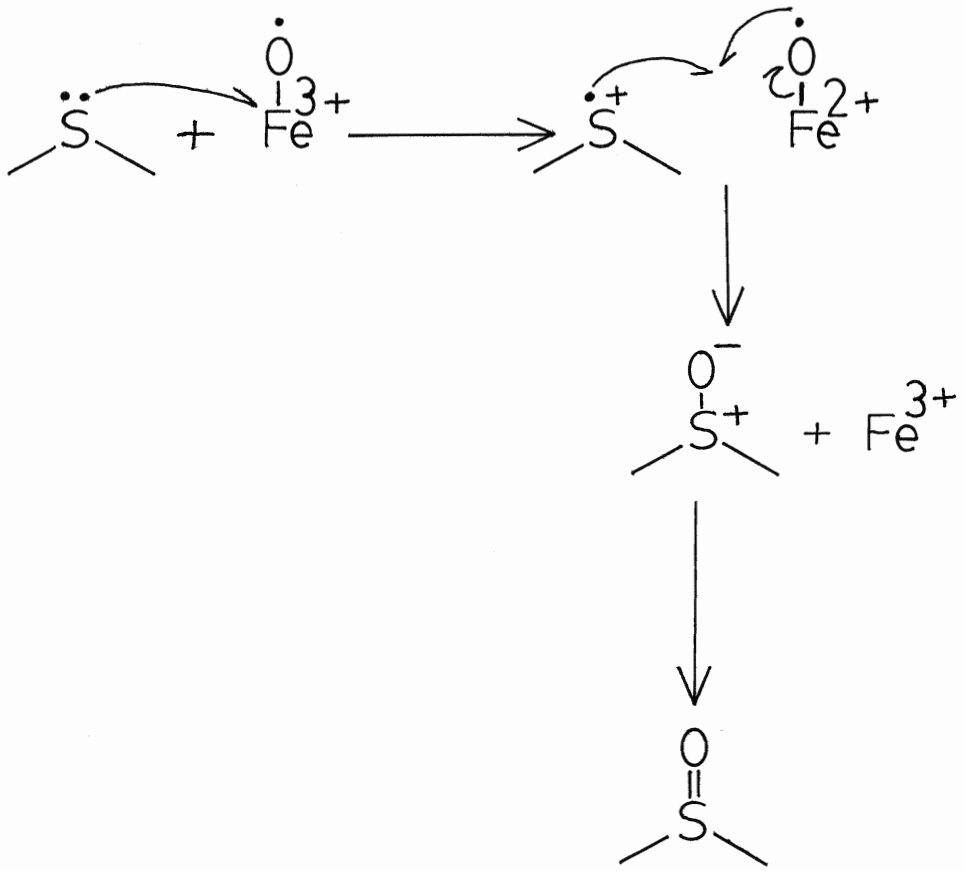
In other experiments,[114] sulphoxidation of a series of substituted methylphenyl and benzylmethyl sulphides have been investigated. The results thus obtained revealed that the oxygen incorporated in the enzymic reaction is derived exclusively from the atmosphere and not from water. The data obtained in these experiments were interpreted in terms of a mechanism of oxidation at sulphur involving an electrophilic attack on the sulphide sulphur by the activated iron-oxygen complex, followed by conversion of the resulting sulphur cation to sulphoxide.

The present study is concerned with transformations catalysed by cytochrome P-450. A variety of fungi known to use cytochrome P-450 dependent monooxygenases have been used.

The first part of this study deals primarily with the metabolism of common environmental pollutants by cytochrome P-450 containing microorganisms.

In the second part, the mechanism of cytochrome P-450 catalysed

Figure 30. Proposed mechanism for sulphoxidation by Cytochrome P-450



hydroxylations has been investigated. Ethylbenzene with a variety of electron donating and withdrawing substituents at para position has been used as a substrate. If the hydroxylation proceeds by concerted mechanism (Fig. 24 A), then there should be no effect of para substituent on the optical purity of product formed, but if stepwise mechanism (Fig. 24 B) is operative and a free radical cation is formed as an intermediate, then the enantiomeric excess may depend upon the stability of intermediate which ultimately depends upon the electronic character of the para substituent. In this way it is hoped that some insight of the mechanism of cytochrome P-450 catalysed hydroxylation could be obtained.

EXPERIMENTAL

Apparatus, Material, and Methods

Melting points were determined on a GallenKamp apparatus and are uncorrected. Ultraviolet spectra were obtained on a Hitachi Perkin-Elmer 124 spectrophotometer with HPLC grade methanol as solvent and reference. Infrared spectra were recorded on a Analect FX6260 FT IR spectrophotometer. Proton NMR spectra were recorded at 60 MHz with a Bruker WP-60 FT NMR spectrometer or at 80 MHz on a Bruker WP-80 CW NMR spectrometer using $CDCl_3$ or acetone-d_6 as solvent and TMS as internal standard . ^{13}C NMR spectra were obtained at 15.18 MHz with a Bruker WP-60 FT NMR spectrometer or at 100 MHz with a Bruker WH-400 FT NMR spectrometer or at 20.115 MHz with Bruker WM-80 FT NMR spectrometer using $CDCl_3$ as a solvent and TMS as internal standard unless otherwise stated. ^{19}F NMR spectra were recorded at 56 MHz with a Bruker WP-60 FT NMR spectrometer using CCl_4 as solvent and $CFCl_3$ as internal standard. Enantiomeric ratios were determind in CCl_4 in the presence of (tris[3-(heptafluoropropylhydroxymethylene)- d -camphorato]europium(III). Mass spectra were obtained with an AEI MS30 double focusing mass spectrometer interfaced to a Kratos DS-55 data system. EI mass spectra were obtained at 70 eV with a 4K accelerating voltage. Column chromatography was performed on Merk silica gel (60-200 mash), and thin layer chromatography on Merk silica gel 60F-254 (0.2 mm thick). The plates were examined under UV light or were sprayed with a 80% concentrated sulfuric acid/ethanol solution or with a 2% (w/v) methanolic solution of 2,6-dichloroquinone-4-chlorimide (Gibb's

regent) and developed at 110°C. Preparative tlc was performed on Merk silica gel F254 (2.0 mm thick) and was examined under UV light. Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter using a 1 dm cell and 95% ethanol as a solvent. The incubation flasks were kept on a New Brunswick rotary shaker (23-25°C, 150-250 rpm).

Stock cultures of Cunninghamella elegans * ATCC 26269 were maintained on potato dextrose agar slopes. Rhizopus arrhizus ATCC 11145 and Mortierella isabellina NRRL 1757 were maintained on 4% malt agar slopes.

Elemental analysis were performed by Guelph Chemical Laboratories Ltd., Guelph, Ont.

* This was the name of fungi when the work described herein was started but after one year it changed to Cunninghamella echinulata var. elegans . However, in the text of this thesis the fungi is refered to by its old name.

PART ONE

Synthesis of Substrates

Most of the compounds used in the present study were either obtained commercially or were generously given by some one, so their synthesis will not be discussed here, only their spectral and physical data are given here. However, appendix I contains the information in the form of a chart which describes the source from which they were obtained and in some cases the literature reference of their preparation.

Dibenzothiophene , 1

M.P. 97-100°C. Its ^{1}H NMR spectrum included signals at δ: 7.3 (2H,m,C-2,3,6,&7H), 7.75 (2H,m,C-4&5H), and 8.05 (2H,m,C-1&8H). UV spectrum, λ_{max} at 257, 282, 310, and 325 nm. Mass spectral data, m/e (%): 184 (100,$M^{+\cdot}$), 152 (11), 139 (22), 113 (4), 95 (5), 79 (4), 74 (5), 69 (7). The ^{13}C NMR spectral data is listed in Table 2.

Dibenzopyrrole (Carbazole) , 2

M.P. 242-243°C. The ^{1}H NMR[151](acetone-d_6) included peaks at δ: 7.0-7.5 (6H,m,C-2,3,4,5,6,&7H), 8.05 (2H,d,$J_{1,2}$=7.5Hz,C-1&8),and 10.25 (1H,s,br,NH). Mass spectral data, m/e (%): 167 (100,$M^{+\cdot}$), 166 (18), 140 (11), 139 (14), 113 (3), 87 (3), 83 (6), 75 (3), 63 (6). The ^{13}C NMR spectral data is given in Table 2.

Fluorene , 3

M.P. 116-117°C. The ^{1}H NMR included peaks at δ: 3.9 (2H,s),

7.3 (4H,m), 7.5 (2H,m), and 7.75 (2H,m). Mass spectral data, m/e (%): 165 (100,$M^{+\cdot}$), 163 (16), 139 (16), 115 (17), 87 (6), 82 (9), 81 (5), 74 (5). The ^{13}C NMR spectral data is listed in Table 2.

9-Fluorenone , 4

M.P. 82-83°C. The ^{1}H NMR contained peaks at δ: 7.25 (2H,m), 7.6 (2H,m). Infrared spectrum, ν_{max}: 1717 cm^{-1} (cyclic ketone), and 3012 cm^{-1} (unsaturated C=C). The mass spectral data, m/e (%): 180 (100,$M^{+\cdot}$), 152 (30), 151 (16), 150 (11), 126 (6), 76 (18), 75 (6), 63 (7). The ^{13}C NMR spectrum is assigned in Table 2.

Acridine , 5

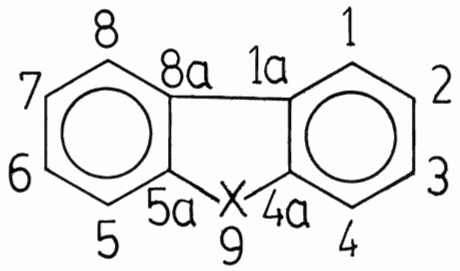
M.P. 111°C. The ^{1}H NMR included peaks at δ: 7.55 (2H,m), 7.7 (2H,m), 8.0 (2H,d), 8.2 (2H,d), 8.75 (2H,s). Mass spectral data, m/e (%): 179 (100,$M^{+\cdot}$), 178 (28), 153 (26), 152 (11), 151 (10), 126 (5), 89 (11), 75 (8). The ^{13}C NMR spectral data is given in Table 2.

Phenanthridine , 6

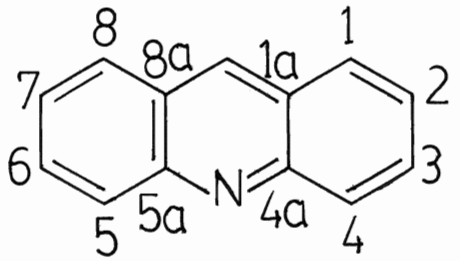
M.P.105-106°C. The ^{1}H NMR included peaks at δ: 7.4-8.05 (5H,m), 8.1-8.6 (3H,m), and 8.27 (1H,s). Mass spectral data, m/e (%): 179 (100,$^{+\cdot}$), 178 (21), 177 (10), 151 (13), 126 (3), 90 (12), 77 (4), 75 (7).

1:10-Phenanthroline , 7

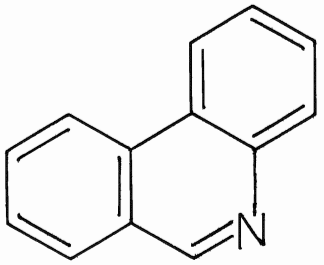
M.P.117°C. The ^{1}H NMR showed signals at δ: 7.5 (2H,t), 7.7 (2H,s), 8.15 (2H,dd), and 9.18 (2H,dd). Mass spectral data, m/e (%): 180 (100,$M^{+\cdot}$), 179 (40), 154 (15), 153 (12), 152 (7), 127 (5), 90 (11), 63 (7). The ^{13}C NMR spectral data is listed in Table 1.



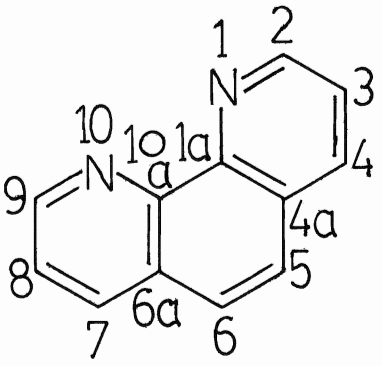
1 X = S
2 X = NH
3 X = CH
4 X = CO



5



6



7

Benz[a]anthracene , 8

The ^{1}H NMR included the signals at δ : 7.33-7.78 (8H,m), 7.85-8.08 (2H,m), 8.2 (1H,s), and 9.9 (1H,s). Mass sectral data, m/e (%): 228 (100,$M^{+\cdot}$), 227 (12), 149 (40), 101 (29), 88 (34), 71 (52), 57 (88), 55 (80). The ^{13}C NMR spectral data is given in Table 1.

Benz[c]acridine , 9

The ^{1}H NMR included peaks at δ : 7.38-8.0 (8H,m), 8.33 (2H,dd), and 8.43 (1H,s). Mass spectral data, m/e (%): 229 (100,$M^{+\cdot}$), 228 (36), 207 (16), 149 (19), 114 (23), 98 (24), 83 (40), 81 (36).

Dihydronorhorman , 10

It was synthesized by the series of reactions illustrated in Figure 31. Mass spectral data, m/e (%): 170 (66,$M^{+\cdot}$), 169 (100), 143 (8), 142 (21), 115 (140), 85 (7), 74 (15), 59 (24).

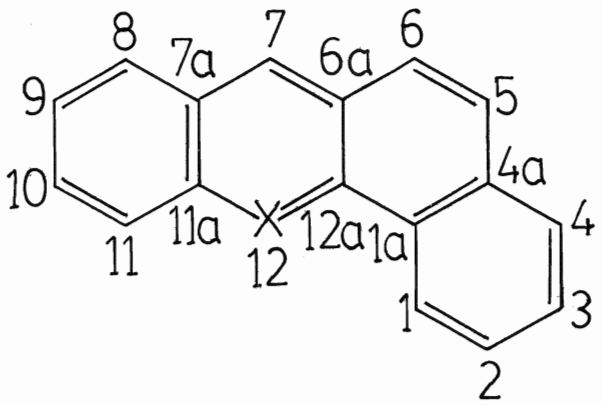
Attempted synthesis of 9H-Pyrido [3,4-b] indole , 11

Three different methods for the synthesis of title compound were attempted, using 10 as the starting material.

Method 1.

Reaction of 10 with Iodine[154]

The first attempt to synthesise 11 , iodine and sodium acetate were used. 1.5 g of dihydronorharman, 10 was added to 30 mL of ethanol and 0.75 g of iodine + 1.5 g of sodium acetate were dissolved in the reaction mixture. The mixture was refluxed for 4 hours, after this the solvent was evaporated on rotary evaporator. The remaining residue was dissolved in $CHCl_3$ and washed with sodium thiosulphate. The



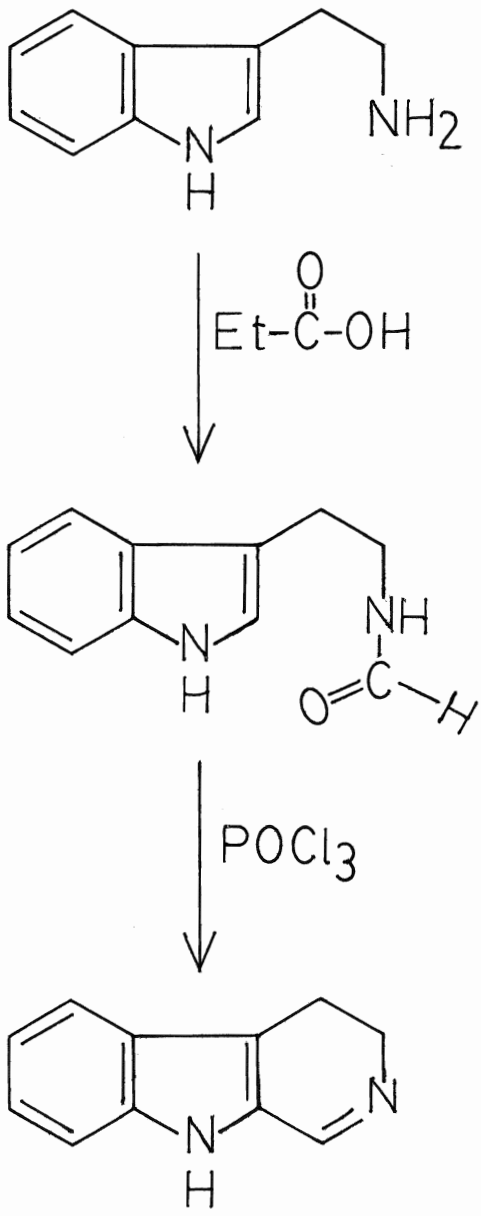
8 X = CH

9 X = N

Table 1. ^{13}C NMR spectra of substrates

Carbon #	Compound	
	7 [152]	8 [153]
1	-	122.8
2	150.6	126.7
3	123.4	126.6
4	136.3	128.5
5	126.8	126.9
6	-	126.9
7	-	127.2
8	-	128.3
9	-	125.6
10	-	125.7
11	-	127.6
12	-	121.4
1a	145.6	130.6
4a	129.0	131.9
6a	-	131.9
7a	-	131.8
11a	-	128.8
12a	-	130.5

Figure 31. Synthesis of Dihydronorharman, 10



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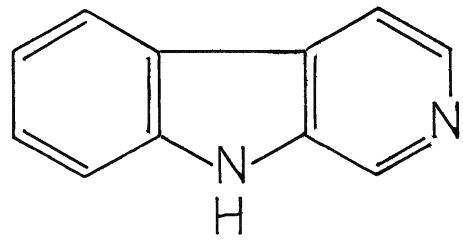


11

compound was extracted with 5% HCl solution. The acidic extract was neutralized by the addition of ammonia and then extracted with ether. The extract was dried over anhydrous sodium sulphate and evaporated to dryness.

The work-up provided 0.95 g of crude extract. Thin layer chromatography (tlc) of the crude extract showed that it contained only one compound with an R_f value identical to that of an authentic sample of 10 . The ^1H NMR spectrum of the extract was also identical to that of 10 .

Method 2.

Reaction of 10 with 2,3-dichloro-5,6-dicyano-1,4-quinone (DDQ)[154]

The second attempt to synthesize 11 , DDQ was used. 0.2 g of dihydronorharman 10 was dissolved in 10 mL of ethanol and 0.2 g of DDQ was added to the mixture. The mixture was stirred at R.T. and tlc was checked at regular intervals. After 48 hours of stirring there was no progress of reaction therefore two drops of conc. HCl were added to the reaction mixture and it was further stirred for 24 hours.

The work-up provided 0.12 g of crude extract. The tlc and ^1H NMR of this extract were identical to that of starting material.

The reaction was repeated using 0.4 g of DDQ but same results were obtaind.

Method 3.

Reaction of 10 with Jones' reagent

0.2 g of dihydronorharman 10 was dissolved in acetone. A

solution of 0.88 g of chromium trioxide in 4.0 mL of 25% (v/v) aqueous H_2SO_4 was added dropwise over a period of five minutes and the reaction was stirred at 5-10°C for 1 hour. The excess of chromic acid was destroyed by isopropanol and a saturated solution of sodium acetate. The mixture was poured into water and extracted with ether. The ethereal layer was washed with water till neutral, dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure.

This work-up gave 0.1 g of crude extract. TLC and ^1H NMR of this sample looked identical to that of a genuine sample of 10 .

All the methods for the synthesis of 11 were obviously unsuccessful and therefore were abandoned.

Triphenylphosphine , 12

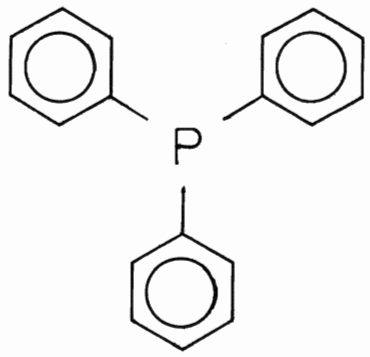
The title compound was purified by recrystallizing the commercially available impure sample in ethanol. After recrystallization, the pure compound was obtained in 80% recovery. M.P. 79-80°C. The ^1H NMR showed a peak at δ : 7.25 (15H,d). Mass spectral data, m/e (%): 262 (100,$M^{+\cdot}$), 261 (16), 201 (19), 184 (20), 183 (99), 152 (26), 87 (47), 77 (16).

Dibenzo-1,4-dioxin , 13

The ^1H NMR spectrum included a single peak at δ : 6.75 (8H,s). Mass spectral data, m/e (%): 184 (100,$M^{+\cdot}$), 155 (5), 128 (16), 127 (5), 102 (5), 92 (18), 64 (40), 63 (4).

5H-Dibenzo[a,d]cyclohepten-5-one , 14

M.P. 88-89°C. The ^1H NMR spectrum included signals at δ : 6.76



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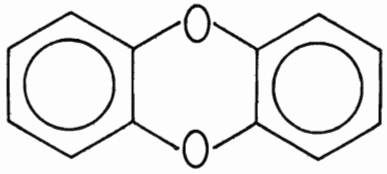


13

(2H,s), 7.1-7.55 (6H,m), and 8.15 (2H,m). Mass spectral data, m/e (%): 206 (97,$M^{+\cdot}$), 179 (15), 178 (100), 176 (35), 151 (13), 89 (21), 88 (23), 76 (29).

10,11-Dihydro-5H-dibenzo[a,d]cyclohepten-5-one , 15

M.P. 32-34°C. The ^{1}H NMR spectrum included peaks at δ: 3.0 (4H,s), 6.93-7.40 (6H,m), and 7.95 (2H,m). Mass spectral data, m/e (%): 208 (100,$M^{+\cdot}$), 207 (45), 193 (8), 179 (55), 178 (53), 165 (26), 152 (10), 89 (24).

Thianaphthene , 16

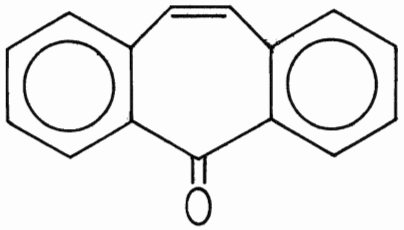
M.P. 29-32°C. The ^{1}H NMR spectrum contained signals at δ: 7.3 (4H,m), and 7.75 (2H,m). Mass spectral data, m/e (%): 134 (100,$M^{+\cdot}$), 133 (5), 108 (4), 90 (10), 89 (10), 69 (4), 67 (5), 63 (5). The ^{13}C NMR spectral data is presented in Table 4.

Alpha-Terthienyl (α-T) , 17

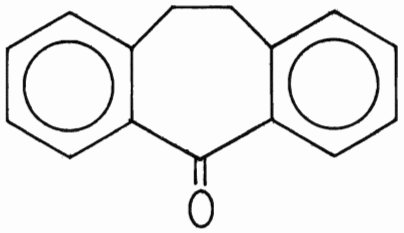
M.P. 91-92°C. The ^{1}H NMR showed peaks at δ: 6.95 (2H,m), 7.1, and (6H,m). Infrared spectrum contained abrorptions at ν_{max}: 3060, 1440, 860, 795, 685, and 675 cm^{-1}. UV spectrum, λ_{max}: 200, 250, and 350 nm. Mass spectral data, m/e (%): 248 (100,$M^{+\cdot}$), 247 (12), 246 (12), 203 (33), 171 (16), 124 (30), 121 (34), 96 (15).

Thiochroman-4-one , 18

B.P. 154°C. The ^{1}H NMR spectrum showed peaks at δ: 2.78-3.33 (4H,m), 6.95 (3H,m), and 8.0 (1H,d). Infrared spectrum, ν_{max}: 1682 cm^{-1} (Cyclic ketone). Mass spectral data, m/e (%): 164



14



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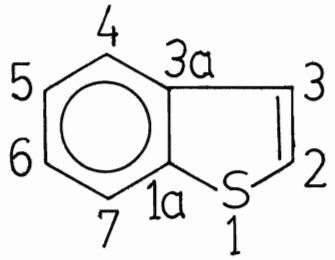

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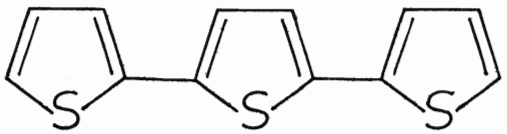


17

(53,$M^{+\cdot}$), 137 (9), 136 (100), 108 (31), 69 (11). The ^{13}C NMR spectral data is given in Table 4.

2-Phenylthiochroman-4-one , 19

Mass spectral data, m/e (%): 222 (100), 221 (30), 134 (35), 165 (10), 120 (79), 102 (13), 97 (16), 92 (38).

Thioxanthan-9-one , 20

M.P. 210-213°C. The ^{1}H NMR spectrum contained peaks at δ: 7.5 (6H,m), and 8.6 (2H,m). Infrared spectrum, ν_{max}: 1642 cm^{-1} (Carbonyl group). Mass spectral data, m/e (%): 212 (100,$M^{+\cdot}$), 184 (38), 152 (7), 139 (13), 92 (14), 79 (10), 69 (9), 58 (7). The ^{13}C NMR spectral data is given in Table 4.

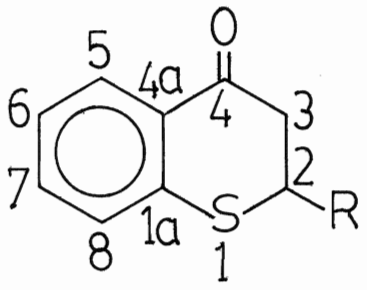
1-Methylthioxanthone , 21

M.P. 93-95°C. The ^{1}H NMR spectrum showed signals at δ : 2.85 (3H,s), 7.05-7.3 (6H,m), and 8.35 (1H,m). Infrared spectrum, ν_{max}: 1641 cm^{-1} (C=O). Mass spectral data, m/e (%): 226 (100,$M^{+\cdot}$), 225 (46), 197 (42), 165 (13), 152 (10), 113 (10), 94 (13), 78 (10). The ^{13}C NMR spectrum is assigned in Table 6.

2-Methylthioxanthone , 22

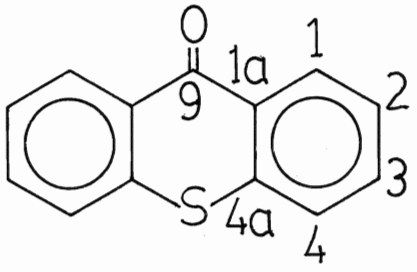
M.P. 122-124°C. The ^{1}H NMR spectrum included peaks at δ: 2.45 (3H,s), 7.18-7.63 (5H,m), 8.3 (1H,s), and 8.55 (1H,d). Infrared spectrum, ν_{max}: 1632 cm^{-1} (C=O). Mass spectral data, m/e (%): 226 (100,$M^{+\cdot}$), 225 (12), 197 (32), 165 (9), 113 (9), 94 (16), 78 (9), 69 (19). The ^{13}C NMR is assigned in Table 6.

3-Methylthioxanthone , 23



18 R = H

19 R = CH_3



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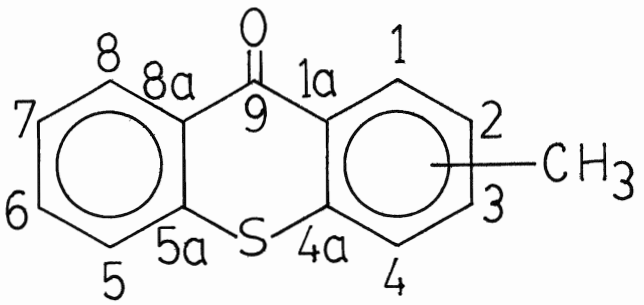


21 1_Methyl

22 2_Methyl

23 3_Methyl

24 4_Methyl

M.P. 115-116°C. The ^{1}H NMR spectrum included signals at δ: 2.4 (3H,s), 7.08-7.58 (5H,m), and 8.4 (2H,m). IR spectrum, ν_{max}: 1637 cm^{-1} (C=O). Mass spectral data, m/e (%): 226 (100,$M^{+\cdot}$), 198 (17), 197 (32), 165 (8), 152 (6), 94 (10), 78 (10), 69 (12). The ^{13}C NMR spectrum is assigned in Table 6.

4-Methylthioxanthone , 24

M.P. 146-148°C. The ^{1}H NMR spectrum showed peaks at δ: 2.45 (3H,s), 7.15-7.6 (5H,m), 8.45 (2H,m). Infrared spectrum, ν_{max}: 1632 cm^{-1} (C=O). Mass spectral data, m/e (%): 226 (100,$M^{+\cdot}$), 225 (13), 197 (38), 165 (71), 152 (8), 98 (8), 94 (17), 77 (11). The ^{13}C NMR spectrum is assigned in Table 6.

Canadine , 25

Mass spectral data,m/e (%): 339 (49,$M^{+\cdot}$), 338 (28), 308 (9), 278 (3), 174 (23), 165 (24), 164 (100), 149 (83).

Tetrahydropalmatine , 26

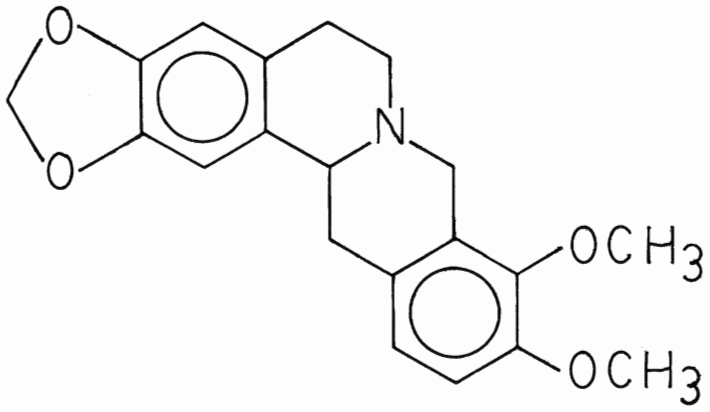
Mass spectral data, m/e (%): 355 (58,$M^{+\cdot}$), 354 (46), 324 (12), 190 (30), 165 (24), 164 (100), 149 (88), 121 (24).

Corydaline , 27

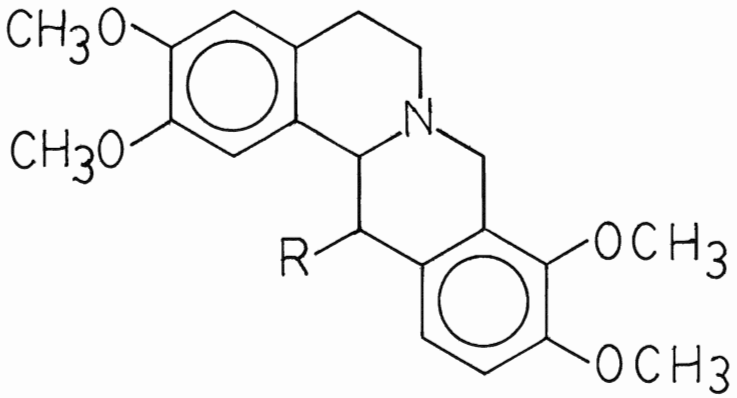
Mass spectral data, m/e (%): 369 (26,$M^{+\cdot}$), 368 (9), 354 (8), 179 (14), 178 (100), 163 (23), 135 (10), 91 (9).

Ethylbenzene , 28a

B.P. 136°C. The ^{1}H NMR spectrum contained signals at δ: 1.25 (3H,t,J=6Hz), 2.6 (2H,q,J=6Hz), and 7.1 (5H,s). Mass spectral data, m/e (%): 106 (37,$M^{+\cdot}$), 105 (8), 92 (8), 91 (100), 77 (9), 65 (8), 51



25



26 R = H

27 R = CH_3

(10).

p-Ethyltoluene , 28b

B.P. 162°C. The ^{1}H NMR spectrum included signals at δ : 1.25 (3H,t,J=6Hz), 2.35 (3H,s), 2.6 (2H,q,J=6Hz), and 7.1 (4H,s). Mass spectral data, m/e (%): 120 (32,$M^{+\cdot}$), 119 (4), 106 (15), 105 (100), 103 (5), 91 (9), 79 (8), 77 (9).

p-Diethylbenzene , 28c

B.P. 184°C. The ^{1}H NMR showed the signals at δ: 1.2 (6H,t,J=6Hz), 2.58 (4H,q,J=6Hz), and 7.05 (4H,s). Mass spectral data, m/e (%): 134 (45,$M^{+\cdot}$), 120 (10), 109 (100), 117 (10), 105 (74), 103 (8), 91 (23), 77 (11).

p-Methoxyethylbenzene , 28d

The ^{1}H NMR showed the peaks at δ : 1.2 (3H,t,J=6Hz), 2.53 (2H,q,J=7Hz), 3.65 (3H,s), and 6.73, 7.0 (4H,AB quartet,J=8Hz). Mass spectral data, m/e (%): 136 (29,$M^{+\cdot}$), 124 (4), 122 (9), 109 (15), 105 (7), 91 (7), 77 (9).

p-Fluoroethylbenzene , 28e

The ^{1}H NMR showed the signals at δ : 1.18 (3H,t,J=7Hz), 2.3 (2H,q,J=7Hz), 6.78-7.28 (4H,m). Mass spectral data, m/e (%): 124 (28,$M^{+\cdot}$), 120 (5), 110 (7), 109 (100), 105 (13), 103 (6), 83 (7), 51 (4).

p-Chloroethylbenzene , 28f

The ^{1}H NMR included the signals at δ : 1.18 (3H,t,J=7Hz), 2.58 (2H,q,J=7Hz), and 7.08 (4H,AB quartet,J=8Hz). Mass spectral data,

m/e (%): 142 (12), 140 (38), 127 (32), 125 (100), 103 (10), 89 (9), 77 (10).

p-Cyanoethylbenzene, [155] 28g

2.2 g (1.7 mL; 0.012 mole) of p-bromoethylbenzene and 1.18 g (0.0132 mole) of cuprous cyanide were heated in refluxing N,N-dimethyl formamide (40 mL), under nitrogen for four hours. The cooled reaction mixture was poured into the solution of ferric chloride hydrate (6.0 g) in concentrated hydrochloric acid (2.0 mL) and water (10 mL), heated for about 20 minutes at 50-60°C, brought to room temperature and extracted with ether (4 x 50 mL). The extracts were combined, washed with 5% sodium hydroxide solution (35 mL), water (4 x 50 mL), and dried over anhydrous sodium sulphate. The solvent was removed over rotatry evaporator.

This work-up provided 1.7 g of crude extract. TLC of the extract showed that it contained one major component with an R_f lower than that of starting material. The extract was chromatographed on a column of silica gel (170 g) by stepwise elution from benzene to ether (steps of 5% ether). 1.3 g of pure compound was isolated giving one spot on tlc. Its ^1H NMR spectrum included peaks at δ: 1.25 (3H,t,J=8Hz), 2.7 (2H,q,J=8Hz), and 7.2, 7.5 (4H,AB quartet,J=9Hz). Infrared spectrum, ν_{max}: 2227 cm^{-1} (C≡N). Mass spectral data, m/e (%): 131 (36,$M^{+\cdot}$), 130 (6), 117 (10), 116 (100), 103 (8), 89 (11), 85 (14), 83 (21).

p-Ethylaniline , 28h

The ^{1}H NMR spectrum of the title compound contained signals at δ : 1.08 (3H,t,J=8Hz), 2.45 (2H,q,J=8Hz), 3.33 (2H,br,s), and 6.40, 6.78 (4H,AB quartet,J=8Hz). Mass spectral data, m/e (%): 121 (38,$M^{+\cdot}$), 120 (6), 107 (8), 106 (100), 79 (4), 77 (9), 53 (5).

p-Ethylacetanilide ,[156] 28i

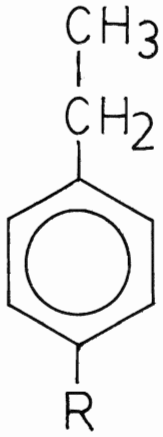
3.27 g (0.027 mL) p-ethylaniline, 2.7 g (2.5 mL; 0.0265 mole) acetic anhydride, and a pinch of zinc dust were placed in 125 mL boiling flask fitted with a condenser. The reaction was boiled gently for one hour, cooled to room temperature and extracted with ether (5 x 50 mL). The extracts were combined, washed with water (3 x 50 mL) and dried over anhydrous sodium sulphate. The solvent was removed on a rotary evaporator and the resulting brown solid on recrystallization from a mixed solvent system of ethanol/water afforded white crystals giving one spot on tlc, (yield 1.28 g; 30%); m.p. 90-92°C. Its ^{1}H NMR included signals at δ : 1.18 (3H,t,J=8Hz), 2.08 (3H,s), 2.55 (2H,q,J=8Hz), and 7.05, 7.33 (4H,AB quartet,J=8Hz). Mass spectral data, m/e (%): 163 (29,$M^{+\cdot}$), 121 (35), 120 (4), 107 (8), 106 (100), 91 (3), 77 (7), 65 (3).

p-Ethylphenol , 28j

M.P. 42-45°C. The ^{1}H NMR Spectrum of the title compound showed signals at δ: 1.15 (3H,t,J=7Hz), 2.55 (2H,q,J=7Hz), 5.5 (1H,s), and 6.65, 7.0 (4H,AB quartet,J=7Hz). Mass spectral data, m/e (%): 122 (38,$M^{+\cdot}$), 108 (5), 107 (100), 77 (8).

n-Propylbenzene , 29

B.P. 159°C. The ^{1}H NMR spectrum included peaks at δ: 0.9



28

a; R = H
b; R = CH_3
c; R = C_2H_5
d; R = OCH_3
e; R = F
f; R = Cl
g; R = CN
h; R = NH_2
i; R = $NHCOCH_3$
j; R = OH

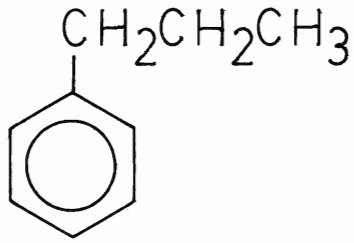
(3H,t,J=6Hz), 1.63 (2H,m), 2.56 (2H,t,J=6Hz), and 7.12 (5H,s). Mass spectral data, m/e (%): 120 (21,$M^{+\cdot}$), 105 (6), 92 (12), 91 (100), 78 (6), 77 (5), 65 (11), 51 (6).

1-(p-methylphenyl)ethanol , 30

p-Methylacetophenone, 35b (10 g; 0.075 mole) and sodium boro-hydride (11.3 g; 0.0297 mole) were dissolved in 40 mL of isopropyl alcohol and the mixture was stirred for 40 hours at room temperature. After this the solvent was evaporated at reduced pressure and 300 mL water was added. The product was extracted with ether (5 x 60 mL). The ethereal layer was washed with water (3 x 300 mL) in order to remove inorganic impurities, and dried over anhydrous sodium sulphate. The solvent was removed on rotary evaporator and 7.8 g (78% yield) product was obtained. TLC indicated that this consisted of a single product. The ^{1}H NMR spectrum included signals at δ : 1.3 (3H,d,J=6Hz), 2.25 (3H,s), 3.25 (1H,br,s), 4.65 (1H,q,J=6Hz), and 7.0 (4H,s). Mass spectral data, m/e (%): 136 (24,$M^{+\cdot}$), 121 (100), 118 (35), 115 (16), 93 (93), 92 (24), 91 (94), 77 (50).

Attempted synthesis of p-Methyl-1-fluoroethylbenzene ,[157] 31

A solution of 1.36 g (0.01 mole) of p-Methyl-1-phenylethanol, 30 in 2 mL $CFCl_3$ was slowly added to a solution of 1.16 g (0.01 mole) diethylaminosulfur trifluoride (DAST) in 5 mL $CFCl_3$ cooled to -78°C. The reaction mixture was warmed to room temperature, mixed with cold water and extracted with ether (4 x 50 mL). The ether extracts were combined, washed with water (4 x 50 mL) and dried over $MgSO_4$. The



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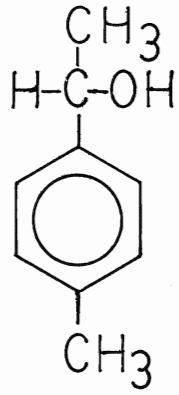

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solvent was evaporated on rotary evaporator.

This work-up provided 0.8 g of crude extract. Thin layer chromatogaphy of the crude extract showed that it contained only one component with an R_f identical to that of starting material, 30 . ^1H NMR was also identical to that of a genuine sample of 30 .

Attempted synthesis of p-Methyl-1,1-difluoroethylbenzene [157], 32

A 4.0 g (4 mL; 0.03 mole) sample of p-Methyl acetophenone, 35b was slowly added to a solution of 4.8 g (3.64 mL; 0.03 mole) of DAST in 15 mL of glyme at 25°C. The reaction was stirred at 85°C, brought to room temperature and then mixed with 50 mL water. The organic layer was extracted with ether (4 x 50mL), washed with water, dried ($MgSO_4$), and solvent was evaporated.

This work-up provided 3.0 g of crude product. TLC of the crude extract indicated that it contained only one component with an R_f value identical to that of starting material. The ^1H NMR spectrum was also identical to that of starting material. It included signals at δ : 2.35 (3H,s), 2.5 (3H,s), and 7.13, 7.75 (4H,AB quartet,J=8Hz).

The reaction was repeated using freshly dried and distilled solvent. The solvent, glyme was dried by treating it with $LiAlH_4$ under nitrogen atmosphere. The same result was obtained. This method for synthesis of 32 was obviously unsuccessful and therefore was abandoned.

Attempted synthesis of 2,2,2,trifluoroethylbenzene , 35

For the synthesis of title compound a series of reactions illustrated in Figure 32, was proposed.

For the synthesis of 34 , trifluoroacetophenone, 36 was treated with $NaBH_4$ under same conditions and same work-up procedure was followed as described for the synthesis of 30 , but the reaction did not occur. Due to the failure of this reaction, proposed scheme for the synthesis of 35 can not proceed further and therefore was abandoned.

Acetophenone , 33a

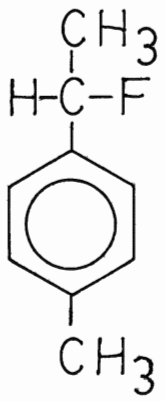
M.P. 19-20°C. Its ^1H NMR spectrum showed peaks at δ : 2.62 (3H,s), 7.45 (3H,m), and 8.0 (2H,m). Mass spectral data, m/e (%): 120 (18,$M^{+\cdot}$), 105 (100), 106 (9), 102 (32), 78 (11), 77 (84), 51 (34), 50 (17).

p-Ethylacetophenone , 33c

B.P. 125°C. The ^1H NMR spectrum of the title compound contained peaks at δ: 1.26 (3H,t,J=7Hz), 2.43 (3H,s), 6.6 (2H,q,J=7Hz), and 7.15, 7.76 (4H,AB quartet,J=8Hz). Mass spectral data, m/e (%): 148 (21,$M^{+\cdot}$), 134 (10), 133 (100), 105 (28), 103 (8), 77 (11), 51 (5).

α,α,α-Trifluoroacetophenone , 36

B.P. 165-166°C. The ^1H NMR spectrum showed signals at δ: 7.6 (3H,m) and 8.05 (2H,d,J=6Hz). Mass spectral data, m/e (%): 174 (8,$M^{+\cdot}$), 106 (7), 105 (100), 78 (6), 77 (9), 74 (5), 51 (35), 50 (14).



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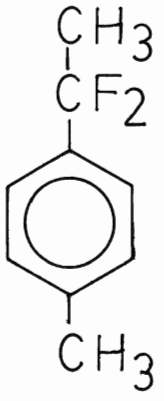

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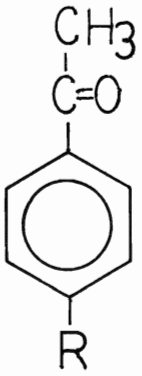

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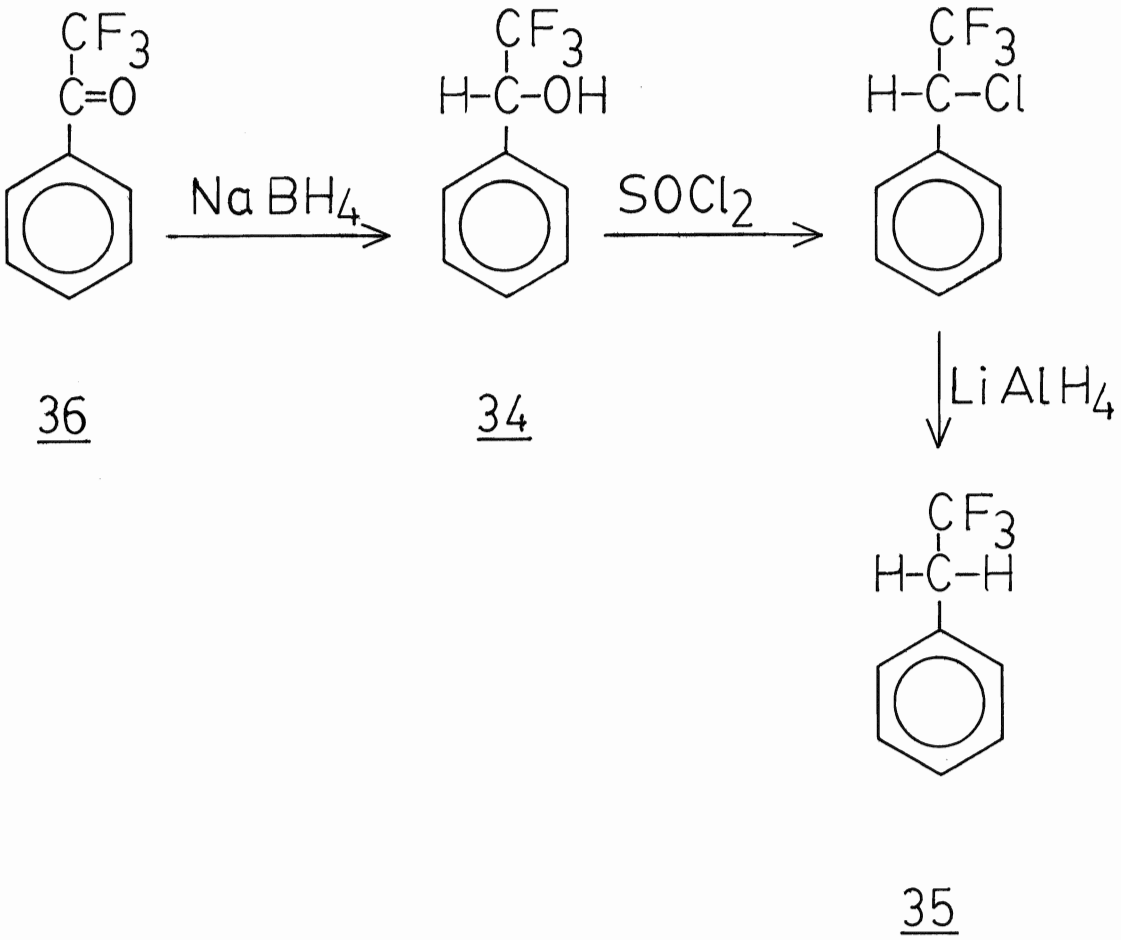
a; R = H

b; R = CH_3

c; R = C_2H_3

Figure 32. Proposed route for the synthesis of 1,1,1-trifluoroethylbenzene, 35



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PART TWO

Microbial Incubations

Section A

Biotransformation of Polycylic Aromatic Compounds by Fungi

1. Incubations with Cunninghamella elegans ATCC 26269 [10,57]

a Preparation and Growth Conditions of Fungal Cultures

The fungi was grown in medium composed of Sabouraud dextrose broth (Difco), 30 g per Litre of distilled water. The fungi was grown in 1 Litre Erlenmeyer flasks, each containing 150 mL of the medium. The flasks were fitted with sponge plugs, sterilized at 15 pounds pressure, 120°C, for 20 minutes, allowed to cool and inoculated (using an inoculation loop) from growing slopes of Cunninghamella elegans . The flasks were then placed on a rotary shaker operating at 250 rpm at 30°C.

b General Design of Experiments

The fungal cultures were grown under the conditions described above for 72 hours. After 72 hours the medium was removed by filtration, and the mycelial pellets were washed 3 times with sterile water, macerated in a Waring blender and resuspended in sterile Sabouraud dextrose broth (150 mL/flask). The substrate dissolved in 95% ethanol (100 mg in 2 mL) was added to each flask. Incubation was then continued under the same conditions as described above for 96 hours. After the incubation period has passed, the mycelium was filtered, and the culture filtrate was extracted three times with ethyl acetate or dichloromethane. The mycelial pellets macerated in a Warying blender with ethyl acetate or dichloromethane and filtered. This process was also repeated three times. The extracts were dried over anhydrous sodium sulphate, and the solvent was removed under reduced pressure. Each of the residues from

the medium and mycelia were examined for metabolic products by thin layer chromatography and subjected to silica gel column chromatography as desired.

A series of control experiments was also performed in some cases as described below.

(I) Incubations with autoclaved fungi were carried out as follows.[154] The washed mycelial pellets were resuspended in medium and then the flasks were autoclaved under the same conditions as described above. After cooling, the substrate was added and subsequent operations were performed as before.

(II) Incubations with attempted induction were tried as follows.[65] The fungus was grown for 48 hours exactly in the same way as in 1a, followed by the addition of inducer substrate (80 μm) and further incubation for 24 hours (induction period). Following an initial three day period of growth and induction, incubation with the substrate was performed under identical conditions as mentioned in 1b and subsequent work-up procedure was adopted.

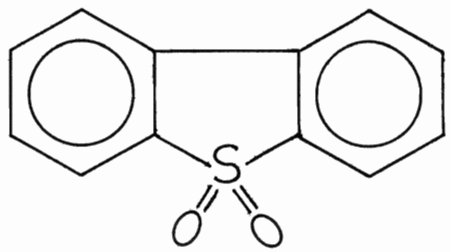
(III) Incubations using cycloheximide as a protein synthesis inhibitor were performed as follows.[158,159] After the standard three day growth period of C. elegans , cyclohexamide (100 μg/ml), and substrate was added to the resuspended fungus in medium and the incubation was continued according to the conditions previously described. When the incubation time had elapsed, the mycelia were again separated from the medium and both were worked-up separately, as described before.

Incubation # 1

Dibenzothiophene , 1

Incubation of this substrate (2.4 g), gave extracts from medium (1.15 g) and mycelium (1.03 g). The former appeared to be a complex mixture (from tlc). The column chromatography of this mixture yielded only starting material in pure form (0.2 g, 8.3% recovery). No other compound can be separated in pure form to be characterized. The latter extract was analysed by tlc in benzene/ether (3:1, v/v) revealed the presence of dibenzothiophene 1 , and two oxidation products (R_f 0.78 and 0.16). Each of the metabolites absorbed U.V. light and showed no reaction with Gibb's reagent. This solid residue was chromatographed on deactivated silica gel (20% water) using benzene-ether and afforded the following components in order of decreasing R_f values.

Dibenzothiophene, 1 (0.4 g, 16.7% recovery), identical with an authentic sample; and dibenzothiophene-9,9-dioxide, 37 (79 mg, 2.8%), m.p. 232-235°C (lit.[160] m.p. 236°C); ^{1}H NMR δ : 7.53 (4H,m), and 7.75 (4H,m); IR ν_{max}: 1165 and 1288 cm^{-1}; UV λ_{max}: 277, 288, 316 nm; MS m/s (%): 216 (100,$M^{+\cdot}$), 187 (47), 171 (19), 168 (50), 160 (40), 149 (38), 136 (42); Anal. calcd. for $C_{12}H_8O_2S$: C 66.66, H 3.70, S 14.81%; found: C 66.45, H 3.88, S 14.57%; The ^{13}C NMR spectrum is assigned in Table 3. The second isolated product was identified as dibenzothiophene-9-oxide, 38 (12 mg, 0.5%), m.p. 189-190°C (lit.[160] m.p. 188°C); ^{1}H NMR δ : 7.5



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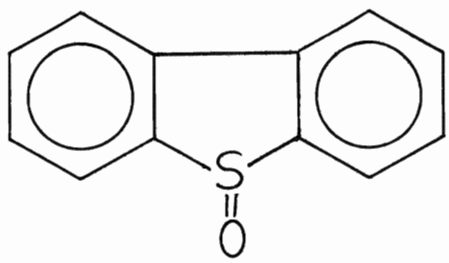

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(4H,m) and 7.85 (4H,m); IR ν_{max}: 1165, 1216, 1270 cm^{-1}; MS m/e (%): 200 (6,$M^{+\cdot}$), 185 (14), 184 (100), 155 (11), 139 (20), 92 (11), 69 (10).

Incubation of 1 with C. elegans was repeated. The fungus was grown by standard procedure as described in section 1a, at the end of growth period the fungal mycelia were filtered off, washed and resuspended, not in medium but in sterile water and incubation was carried out by normal procedure. Incubation of 1.2 g of substrate provided extracts from medium (0.45 g), and mycelium (0.5 g). Thin layer chromatography of crude extracts showed no metabolic product in either of them. Starting material and some highly non-polar endogenous fungal material was detected.

Two control incubations were also performed with this compound.
(i) In the first control experiment, 200 mg of 1 was incubated with an autoclaved culture of C. elegans. This incubation provided extracts which showed only one spot on the tlc with R_f identical to that of an authentic sample of 1. No non-enzymic transformation had taken place.
(ii) In the second control experiment dibenzothiophene was incubated in the presence of cycloheximide. TLC analysis of mycelial extract indicated the formation of products which were characterized as corresponding sulphone, 37 and sulphoxide, 38 .

Incubation # 2

Dibenzopyrrole , 2

Incubation of 2 (1.6 g) provided extracts from mycelium (330 mg) and medium (70 mg). TLC analysis of both the residues in benzene/ether (3:1, v/v) indicated the presence of starting material (R_f 0.78) and one transformation product which absorbed U.V. light and gave a blue colour with Gibb's reagent. The former extract was chromatographed on silica gel by stepwise elution from benzene to ether (increments of 5% ether), the following components were identified : dibenzopyrrole, 2 (150 mg, 9.3) and 2-hydroxydibenzopyrrole, 39 (12 mg, 0.7%), m.p. 253-254°C (lit[161] m.p.260-261°C); ^{1}H NMR (acetone-d_6) δ: 6.83-7.53 (5H,m), 7.83-8.0 (1H,d,$J_{7,8}$=7.5,C-8H), and 7.93 (1H,s,C-1H); IR ν_{max}: 2853, 2924, 2959, 3399 cm^{-1}; MS m/e (%): 183 (100,$M^{+\cdot}$), 182 (14), 154 (19), 127 (11), 91 (21), 77 (14), 63 (7), 57 (7). The ^{13}C NMR spectrum is assigned in Table 3.

The latter extract was subjected to preparative layer chromatography. This provided 5 mg, 0.3% of compound identical in all respects to 39 , obtained earlier.

A control incubation with this substrate, 2 was also carried out. The compound (200 mg) was incubated with the fungus in the presence of cycloheximide (protein synthesis inhibitor). TLC analysis (spray with Gibb's reagent) and mass spectral data of medium extract indicated the the formation of 2-hydroxydibenzopyrrole, 39.

Incubation # 3

Fluorene , 3 .

Incubation of (1.0 g) of title compound gave 0.75 g of extract.

Both the medium and mycelium appeared to be complex mixtures. Column chromatography of both mixtures on silica gel (75 g) using benzene to ether (steps of 10% ether) gradient elution led to the recovery of only starting material (0.4 g, 40%). The recovered substrate was identified by comparison of spectral and physical data of a genuine sample of 3 .

Incubation # 4

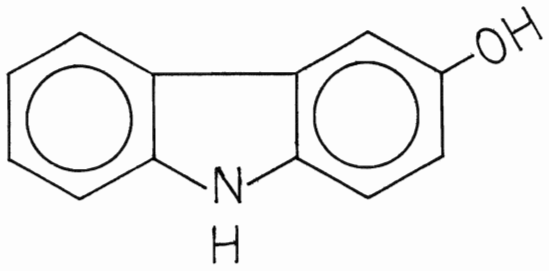
9-Fluorenone , 4

Incubation of 4 (0.5 g) gave extracts from medium (50 mg) and mycelium (310 mg). The latter extract contained natural fungal products, plus 4 as the only readily detectable material. The former extract was chromatographed on silica gel using a benzene to ether solvent gradient elution (steps of 5% ether). The following components were identified: 9-fluorenone, 4 (20 mg), identical to starting material and 9-hydroxy fluorene, 40 (7 mg, 1.4%), m.p. 215-218°C (lit.[162] 153-154°C); ^{1}H NMR δ:5.6 (1H,s), 7.35 (4H,m), and 7.68 (4H,m); IR ν_{max}: 3189 cm^{-1}; MS m/e (%): 182 (77,$M^{+\cdot}$), 181 (100), 166 (51), 164 (12), 153 (21), 151 (12). The ^{13}C NMR spectrum is assigned in Table 3.

Incubation # 5

Acridine , 5

Incubation of 5 (2.4 g) gave crude extract (2.1 g). Both medium and mycelial extracts appeared to be complex mixtures (from tlc). Chromotography of both extracts on silic gel column using benzene to acetone gradient elution (10% increments of acetone) provided starting



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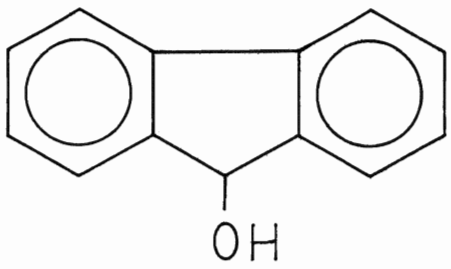

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Table 2. ^{13}C NMR spectra of substrates

Carbon #	Compound				
	1 *	2 *	3 [163]	4	5 [164,165]
1	124.7	120.0	119.8	120.3	130.5
2	122.0	125.4	126.7	134.6	125.7
3	123.0	118.4	126.7	129.0	128.5
4	127.1	110.9	125.0	124.2	129.7
1a	135.1	122.4	141.7	144.4	126.7
4a	138.7	139.7	143.2	134.1	149.3
9	-	-	36.9	193.7	136.1

* Solvent DMSO-d_6

Table 3. Assignment of ^{13}C NMR spectra of Dibenzothiophene-9,9-dioxide, 37 , 2-Hydroxydibenzopyrrole, 39 , and 9-Hydroxyfluorene, 40

Carbon #	Compound		
	37 [a]	39 [a]	40 [b]
1	122.0*	110.7	120.1
2	134.6	150.3	127.9
3	122.7*	104.8	129.2
4	131.0	114.9	125.3
5	-	111.1	-
6	-	117.6	-
7	-	125.0	-
8	-	119.9	-
9	-	-	75.4
1a	137.0	123.0	140.2
4a	137.0	133.7	145.8
5a	-	140.3	-
8a	-	122.2	-

* Chemical shifts marked with asterisks may be interchanged.

a All shifts in ppm relative to solvent DMSO-d_6 at 39.5 ppm.

b All shifts in ppm relative to solvent $CDCl_3$ at 77.2 ppm.

material (0.8 g) plus a metabolic product (180 mg) which appeared to be a hydroxylation product (tlc gave a blue colour with Gibb's reagent). This product still appeared to be contaminated with less polar natural fungal products. The product was therefore rechromatographed by preparative tlc. This provided a metabolite (40 mg) in an impure form. The mass spectrum of this metabolite showed a molecular ion at m/e 195 corresponding to the addition of one oxygen atom to the substrate. Further characterization was not possible due to the limited quantity and the impure state of the material.

A control experiment with this compound was also performed. The substrate 5 (1.0 g) was incubated with the culture of C. elegans which was induced with the same compound. The incubation provided extracts from medium (0.66 g) and mycelium (.036 g). The former contained nonpolar endogenous fungal material in addition to starting material as the only readily detectable compound (tlc) and was not examined further. The latter extract was chromatographed on silica gel (gradient elution from hexane through benzene to ether, steps of 10%) and afforded 5 (0.15 g) and traces of an oxidised metabolite (tlc and mass spectrum) which could not be isolated in sufficient quantity for complete characterization.

Incubation # 6

Phenanthridine , 6

Incubation of 6 (1.6 g) provided 1.4 g of extract. TLC of both the medium and mycelium extracts showed that they contained only

unmetabolized substrate.

Incubation # 7

1:10 Phenanthroline , 7

Incubation of 7 (1.0 g) gave 0.85 g of extract. Both the mycelium and medium extracts gave a spot with R_f identical to that of an authentic sample of 7 and several spots with larger R_f values. There was no indication of more polar oxidation products.

Incubation # 8

Benz[a]anthracene , 8

Incubation of 8 (45 mg) provided 34 mg of crude extract. TLC showed the major component to be the starting material. Further analysis by mass spectroscopy indicated only the starting substrate.

Incubation # 9

Benz[c]acridine , 9

Incubation of 9 (190 mg) gave 150 mg of crude extract. Thin layer chromatography showed the presence of only starting material. The mass spectrum of the crude extract was characteristic of 9 .

Incubation # 10

Triphenylphosphine , 12

Incubation of 12 (0.5 g) gave extracts from medium (0.12 g) and mycelium (0.3 g). The latter contained natural fungal material and 12 as the only readily detectable (tlc) substance and was not examined further. TLC of medium extract revealed the presence of mainly one component with a R_f value lower than the starting substrate.

Recrystallization of the medium extract from ethanol provided 90 mg , (18% yield) of triphenylphosphine oxide, 41 , identical in all respects with commercial sample of 41 , m.p. 153-154°C (lit.[166] m.p. 156-157°C); ^{1}H NMR δ : 7.52 (9H,m), 7.75 (6H,m); IR ν_{max}: 1120, 1178, 1436, 1672, 2929, 2962 cm^{-1}; MS m/e (%): 277 (38,$M^{+\cdot}$), 201 (10), 154 (23), 125 (15), 111 (21), 83 (38), 69 (66), 57 (100).

A control incubation experiment was also carried out with this substrate. The starting material, 12 (200 mg) was incubated with an autoclaved culture of C. elegans. Work-up of this incubation provided, triphenylphosphine oxide, 41 , identified by comparison with a commercial sample.

Incubation # 11

Dibenzo-1,4-dioxin , 13

Incubation of 13 (200 mg) provided 130 mg of crude extract. TLC showed the major component to be the starting substrate. Further analysis by mass spectroscopy showed that only starting material was present.

Incubation # 12

5H-Dibenzo[a,d]cyclohepten-5-one , 14

Incubation of 14 (0.5 g) gave 0.87 g of crude extracts. TLC of these extracts showed that they contained only unchanged starting material. The mass spectrum of the recrystallized extract showed the fragmentation pattern characteristic of the starting substrate, 14 .

Incubation # 13

10,11-Dihydro-5H-dibenzo[a,d]cycloheptan-5-one , 15

Incubation of 15 (0.5 g) provided 0.69 g of crude extract. The crude extract from this culture gave a spot with R_f similar to that of a genuine sample of 15 and several spots with greater R_f values. There was no sign of more polar oxidation product and was therefore, not examined any further.

Incubation # 14

Thianaphthene , 16

Incubation of 16 (1.4 g) gave 1.27 g of crude extract. Thin layer chromatography of this extract revealed the presence of only unmetabolized substrate. The recrystallization of crude extract yielded (0.9 g, 64% recovery) of 16 identical to the starting material.

Incubation # 15

Alpha-Terthienyl , 17

Incubation of 17 (0.8 g) provided 0.94 g of crude extract. The extract appeared (from tlc) to be a complex mixture. The extract was chromatographed on silica gel (gradient elution from hexane through benzene to ether) and gave 17 , (0.55 g, 69% recovery), identified by comparison with an authentic sample. A non-polar component was also obtained. Mass spectral analysis of this species determined that it was not an oxidation product.

Incubation # 16

Thiochroman-4-one , 18

Incubation of 18 (0.4 g) gave extracts from medium (0.24 g) and

mycelium (0.34 g). TLC analysis of both the extracts indicated the presence of more polar oxidation product apart from the starting substrate. Both the extracts were pooled and chromatographed on a silica gel column using benzene-ether-ethyl acetate gradient (increments of 10% polar solvent) as eluting solvents and afforded 18 (0.15 g, 38% recovery) and thiochroman-1-oxide, 42 (0.63 mg, 16% yield). ^{1}H NMR δ : 2.8 (2H,m), 3.35 (2H,m), 7.68 (3H,m), and 8.08 (1H,m); IR ν_{max}: 1030, 1052, and 1689 cm^{-1}; MS m/e (%): 180 (7,$M^{+\cdot}$), 164 (44), 162 (10), 152 (72), 136 (100), 108 (33), 96 (18), 76 (14); The ^{13}C NMR spectrum is assigned in Table 5.

Incubation # 17

2-Phenyl-thiochroman-4-one , 19

Incubation of 19 (0.21 g) gave 0.12 g of extract. Analysis of crude extract by tlc revealed the presence of only starting substrate, which was also supported by mass spectral anlysis.

Incubation # 18

Thioxanthan-9-one , 20

Incubation of 20 (1.0 g) gave an extract (1.29 g). TLC of the crude extract in benzene/ether (3:1, v/v) revealed the presence of starting material 20 and two oxidation products (R_f 0.36 and 0.76). The extract was chromatographed on silica gel by stepwise elution from benzene to ether (increments of 10% ether) and afforded 20 (0.524 g, 53% recovery); thioxanthone-10,10-dioxide, 43 (20 mg, 2% yield), m.p.183-186°C (lit.[167] m.p. 185-187°C); ^{1}H NMR δ : 7.8 (4H,m)

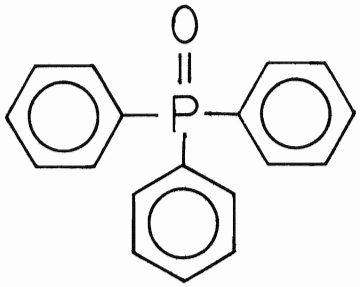
and 8.4 (4H,m); IR ν_{max}: 1054, 1144, 1168, 1294, and 1680; MS m/e (%): 244 (13,$M^{+\cdot}$), 212 (8), 197 (14), 196 (100), 180 (10), 168 (16), 151 (10), 136 (39); The ^{13}C NMR is assigned in Table 5.

The second component isolated was not pure so it was separated by preparative tlc (benzene/ether, 3:1). This provided thioxanthone-10-oxide, 44 (5 mg, 0.5% yield), m.p. 199-201°C (lit.[167] m.p. 200-204°C); ^1H NMR δ: 7.7 (4H,m) and 8.2 (4H,m); IR ν_{max}: 1038, 1264, and 1667 cm^{-1}; MS m/e (%): 228 (1.4 ,$M^{+\cdot}$), 212 (100), 184 (39), 139 (14), 104 (15), 83 (15).

Incubation # 19

1-Methylthioxanthone , 21

Incubation of 21 (0.4 g) provided an extract (0.35 g) which was chromatographed on silica gel column using benzene to ether solvent gradient (steps of 10% ether) to give 1-Methylthioxanthone, 21 (0.09 g, 23% recovery) identical with the starting material; 1-Methylthioxanthone-10,10-oxide, 45 (10 mg, 2.5% yield), m.p. 200-202°C (lit.[168] m.p. 201-203°C); ^1H NMR δ: 2.79 (3H,s), 7.43-7.78 (4H,m), and 7.93-8.18 (3H,m); IR ν_{max}: 1125, 1151, 1308, and 1672 cm^{-1}; MS m/e (%): 258 (99,$M^{+\cdot}$), 226 (77), 210 (68), 209 (19), 197 (27), 181 (20), 165 (100), 149 (19); ^{13}C NMR spectrum is assigned in Table 7; and 1-Hydroxymethylthioxanthone-10-oxide, 49 , (28 mg, 7% yield after recrystallization from hexane), m.p. 166-168°C; ^1H NMR δ: 3.4 (1H,br,s,exchanges D_2O,OH), 4.8 (2H,q,J=12Hz), 7.5-7.85 (4H,m), and 7.9-8.23 (3H,m); IR ν_{max}: 1028, 1052, 1660, and



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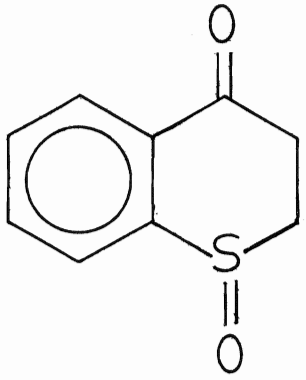

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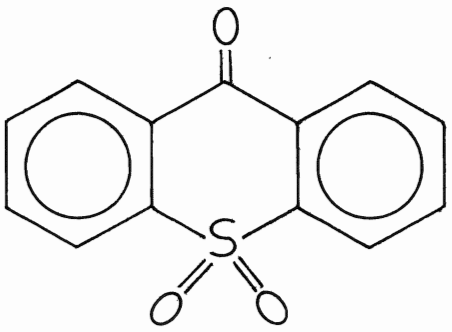

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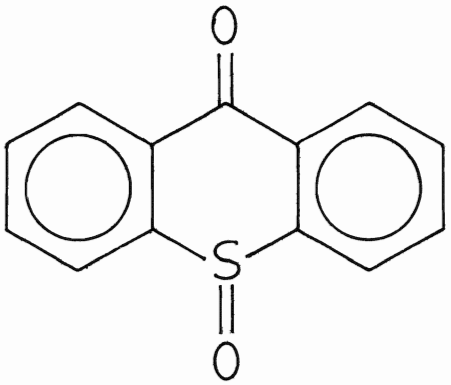

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Table 4. ^{13}C NMR spectra of substrates

Carbon #	Compound		
	16 [169]	18 [170]	20 [171]
1	-	-	129.9
2	122.6	26.6	126.0*
3	126.3	39.5	132.3
4	124.3	193.8	126.3*
5	123.8	129.1	-
6	123.9	124.9	-
7	124.4	133.1	-
8	-	127.5	-
9	-	-	180.0
1a	140.1*	142.1	129.3
3a	139.9*	-	-
4a	-	130.9	137.3

* Chemical shifts marked with asterisks may be interchanged.

Table 5. Assignment of ^{13}C NMR spectra of Thiochromanone-1-oxide, 42 and Thioxanthone-10,10-dioxide, 43

Carbon #	Compound	
	42 [169]	43 [a]
1	-	129.2
2	46.8	134.7
3	30.4	133.2
4	192.0	123.6
5	129.1*	-
6	132.2	-
7	134.6	-
8	128.5*	-
9	-	176.7
1a	145.7	130.6
4a	129.7	140.4

* Chemical shifts marked with asterisks may be interchanged.

a All shifts in ppm relative to $CDCl_3$ at 77.0 ppm.

3377 cm^{-1}; MS m/e (%): 258 (7,$M^{+\cdot}$), 141 (30), 224 (99), 212 (71), 196 (100), 184 (58), 152 (76). An accurate mass/atomic composition spectral analysis was also carried out. The determined mass (258.0363) was in close agreement with the calculated mass of 258.0350 for $C_{14}H_{10}O_3S$ with a deviation of 1.3 mmu. The ^{13}C NMR spectrum of the compound was too complex (perhaps due to the decomposition of compound in $CDCl_3$) to aid in structure determination, however, the peak for methyl carbon was absent and a peak at 64.5 ppm corresponding to benzylic carbon with hydroxyl group was observed. R_f values: Chloroform/Methanol (95:05, v/v), 0.76; Benzene/ether (75:25, v/v), 0.68

Incubation # 20

2-Methylthioxanthone , 22

Incubation of 22 (0.5 g) gave extract (0.61 g) which was chromatographed on silica gel column by stepwise elution from benzene to ether (increments of 10% ether) to give 22 , (0.17, 34%) identical in all respects with starting material and 2-Methylthioxanthone-10,10-dioxide, 46 (25 mg after recrystallization from Chloroform-Hexane, 5% yield), m.p. 203-205°C (lit.[168] m.p. 204-206°C); ^1H NMR δ : 2.38 (3H,s), 7.45-7.93 (4H,m), 7.98 (1H,s), and 8.05-8.38 (2H,m); IR ν_{max}: 1125, 1146, 1160, 1291, 1672 cm^{-1}; MS m/s (%): 258 (34,$M^{+\cdot}$), 226 (9), 210 (100), 209 (12), 181 (18), 165 (29), 150 (11), 136 (25); The ^{13}C NMR spectrum is assigned in Table 7.

Incubation # 21

3-Methylthioxanthone , 23

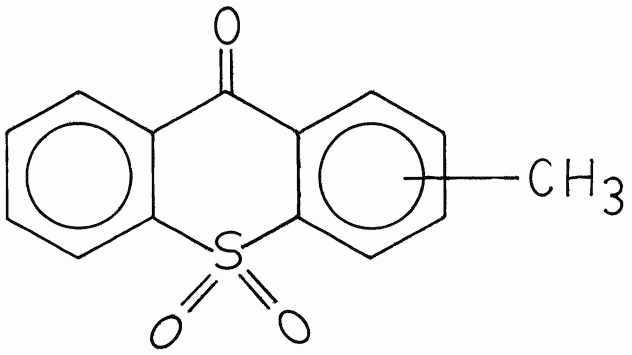
Incubation of 23 (0.5 g) gave an extract (0.66 g) which was applied to the top of a column of silica gel in benzene. The column was eluted with ether-benzene (increments of 10% ether) to give in order of elution, 23 (0.36 g, 72% recovery), identical with starting substrate; 3-Methylthioxanthone-10,10-dioxide, 47 (22 mg, 4.4%), m.p. 205-206°C (lit.[168] m.p. 206-207°C); ^{1}H NMR δ: 2.48 (3H,s), 7.39-7.80 (2H,m), 7.85 (1H,s), and 7.98-8.38 (4H,m); IR ν_{max}: 1155, 1299, 1318, and 1674 cm^{-1}; MS m/e (%): 258 (39,$M^{+\cdot}$), 210 (100), 181 (17), 165 (34), 150 (19), 136 (19); ^{13}C NMR spectrum is assigned in Table 7; and 220 mg of a substance which appeared to be an oxidation product (tlc, ms, and nmr). However it was not pure, so the column chromatography was repeated using the same solvent system as above. This provided (30 mg, 6%, m.p. 159-160°C) of a product tentatively assigned the structure, 3-Hydroxymethylthioxanthone, 50 ; ^{1}H NMR δ: 4.75 (2H,s), 7.48 (5H,m), and 8.5 (2H,m); IR ν_{max}: 1632, 2917, and 3401 cm^{-1}; MS m/e (%): 242 (100,$M^{+\cdot}$), 241 (19), 226 (11), 211 (13), 197 (14), 183 (11). This incubation product was still impure giving one other spot on tlc with R_f higher than that of 50 or starting material 23 . The presence of this impurity meant that the elemental analysis of the product was not helpful and m.p. was not reliable. However, elemental analysis is listed here. Anal. calculated for $C_{14}H_{10}OS$: C 69.42, H 4.13, S 13.22%; Found: C 67.37, H

6.10, S 13.44

Incubation # 22

4-Methylthioxanthone , 24

Incubation of 24 (0.4 g) provided an extract (0.49 g) which appeared (tlc) to contain one major component, more polar than starting material. In order to isolate this species, the crude extract was chromatographed on silica gel using a benzene to ether solvent gradient (steps of 10% ether). The chromatography afforded in order of decreasing R_f, 24 (50 mg, 12.5% recovery) identical in all respects with starting material, and 4-Methylthioxanthone-10,10-dioxide, 48 (40 mg, 10% yield), m.p. 178-180°C (lit.[168] m.p. 181-182°C); ^{1}H NMR δ : 2.83 (3H,s), 7.5 (2H,d), 7.6-7.88 (2H,m), and 7.95-8.33 (3H,m); IR ν_{max}: 1117, 1136, 1153, 1294, 1308, and 1672 cm^{-1}; MS m/e (%): 258 (100,$M^{+\cdot}$), 241 (3), 226 (16), 210 (99), 184 (9), 181 (21), 165 (59); ^{13}C NMR is assigned in Table 7.

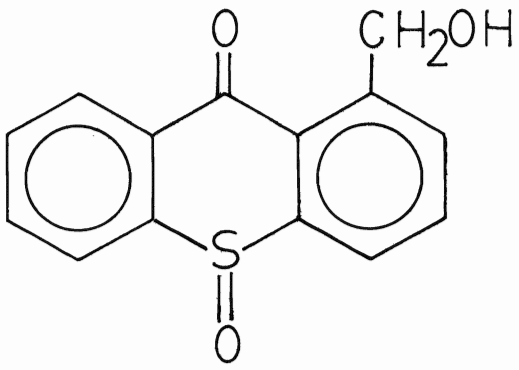


45 1_Methyl

46 2_Methyl

47 3_Methyl

48 4_Methyl



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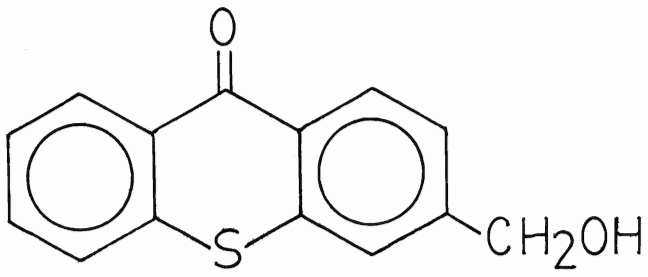

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Table 6. Assignment of ^{13}C NMR spectra of Methyl substituted Thioxanthones.[a]

Carbon #	Compound			
	21	22	23	24
1	144.0	129.8*	129.9	127.7
2	129.8	136.3	126.3*	126.4
3	131.2*	133.6	144.4	133.4
4	124.4	126.0	127.9	134.0
5	126.2	126.0	126.0*	125.6
6	131.8*	132.0	132.1	132.2
7	125.3	126.0	125.8*	126.4
8	130.4	129.6*	129.9	129.7
9	182.6	180.0	179.8	180.4
1a	129.5	129.1	127.2	129.0
4a	138.7[b]	134.2	137.4	Not Seen
5a	138.7[b]	137.5	137.4	136.8
8a	124.6	129.1	129.5	129.0
Methyl	24.9	21.2	21.7	19.4

* Chemical shifts marked with asterisks may be interchanged.

a All shifts in ppm relative to $CDCl_3$ at 77.2 ppm.

b Very weak.

Table 7. Assignment of ^{13}C NMR spectra of Methyl substituted Thioxanthone-10,10-dioxdies[a]

Carbon #	Compound			
	45	46	47	48
1	142.9	129.4*	129.1*	127.3
2	137.1	144.3	134.5	138.0
3	133.0*	135.3**	146.5	132.9
4	122.0	123.6***	123.6**	137.1
5	123.0	123.5***	123.5**	123.4
6	133.3*	133.1	133.2	132.2
7	133.5*	134.6**	134.0	134.8
8	129.0	129.1*	129.3*	128.5
9	181.9	178.6	178.0	178.1
1a	128.7[b]	130.5****	128.1	129.3
4a	142.0[b]	138.2	140.9***	142.2
5a	139.5[b]	141.1	140.8***	138.4
8a	126.4[b]	130.8****	130.6	130.8
Methyl	22.8	21.7	22.0	19.8

* Chemical shifts marked with asterisks may be interchanged.

a All shifts in ppm relative to $CDCl_3$ at 77.0 ppm.

b Very weak.

2. Incubations with Rhizopus arrhizus ATCC 11145 [172]

a. Preparations and Growth Conditions of Fungal Cultures

The growth medium for this fungus composed of 50 g of glucose, 20 g of peptone, and 3 mL of corn steep liquor per litre of distilled water. The fungus was grown utilizing 1-L Erlenmeyer flasks, each containing 150 mL of the medium and fitted with sponge plugs. Prior to inoculation with fungi, the medium was sterilized and cooled to room temperature. The flasks were then incubated and grown out at room temperature for 96 hours on a rotary shaker at 180 rpm.

b. General Design of Experiments

Following an initial four day growth period under the conditions described above the mycelia were then separated from the medium by filtration, washed with distilled water, crushed in a Waring blender with distilled water and aliquots were resuspended in distilled water (150 mL in each flask). A solution of substrate (100 mg dissolved in 2 mL of 95% ethanol) was then added to each flask. The incubation with the substrate was carried out for a further 72 hour period at which time the flasks were filtered and worked-up as previously described in 1b. The crude residues were then subjected to chromatographic analysis as required.

Incubation # 23

Dibenzothiophene , 1

Incubation of 1 (1.0 g) provided extracts from medium (0.6 g) and mycelium (0.42 g). The latter contained starting material as the only readily detectable compound (tlc) and was not examined further. The extract from incubation medium was chromatographed on silica gel by stepwise elution from benzene to ether (increments of 10% ether) to give 1 (0.27, 27%), identical with starting substrate, 37 (6 mg, 0.6%), and 38 (2 mg, 0.2%). These isolated products were identified by mp, tlc, and spectral comparison with the compounds obtained from previous incubation (#1).

Incubation # 24

Canadine , 25

Incubation of 25 (0.2 g) gave 0.17 g of extract; tlc and mass spectral analysis indicated the absence of transformation products. Recrystallization from methanol provided only 25 (0.15 g, 75% recovery), identical with starting substrate.

Incubation # 25

Tetrahydropalmatine , 26

Incubation of 26 (0.2 g) gave 0.18 g of extract which gave a spot on tlc with R_f identical to that of a genuine sample of 25 and a couple of spots with higher R_f values. There was no indication of more polar oxidation products. Further analysis by mass spectrometry

revealed the presence of only starting material. Purification afforded only 26 (0.165 g, 82.5% recovery), identical with starting material.

Incubation # 26

Corydaline , 27

Incubation of 27 (0.2 g) gave 0.175 g of crude extract. Thin layer chromatography indicated the only component to be the starting material. The crude extract was further analysed by mass spectrometry which showed that 27 was the only compound present. The extract was recrystallized from methanol to give 27 (0.155 g, 77.5% recovery), identical with starting material.

3. Incubations with Mortierella isabellina NRRL 1757 [114]

a. Preparation and Growth Conditions of Fungal Cultures

The growth medium for the fungus consisted of glucose (40 g), soya bean meal (5 g), yeast extract (5 g), sodium chloride (5 g), and potassium phosphate (dibasic) (5 g) per Litre of distilled water. One hundred and fifty mL of the above medium was placed in one Litre Erlenmeyer flasks, fitted with sponge plugs, sterilized as described in 1a, allowed to cool and then inoculated with Mortierella isabellina from growing slopes, under sterile conditions. The flasks were then placed on a rotary shaker, 150 rev./min. at 25°C.

b. General Design of Experiments

After three days (72 hours) of growth under the conditions mentioned above, the flasks were removed from the shaker, filtered, mycelia washed two times with distilled water and then resuspended in distilled water (150 mL/flask). The substrate dissolved in 95% ethanol (100 mg/2 mL) was added to each flask. Incubation was then continued under the similar conditions as described above for 72 hours. Subsequent work-up was identical to that described for other cultures. The extracts were filtered through α-cellulose, if necessary. The crude residues obtained were then subjected to chromatography as required.

Incubation # 27

Dibenzothiophene , 1

Incubation of 1 (0.5 g) gave extracts from mycelium (0.62 g) and medium (0.070 g). The former contained 1 as the only readily detectable compound. The latter extract was chromatographed on silica gel by stepwise elution from benzene to ether (increments of 10% ether) to give dibenzothiophene, 1 (20 mg, 4%) identical with authentic material; dibenzothiophene-9,9-dioxide, 37 (25 mg, 5% yield), and dibenzothiophene-9-oxide, 38 (5 mg, 1% yield), identified by comparison of spectral and physical properties with products, 37 and 38 obtained from previous incubations.

Incubation # 28

Thioxanthan-9-one , 20

Incubation of 20 (0.9 g) gave extracts from medium (0.5 g) and mycelium (0.8 g). The latter contained starting material as the only readily detectable component (tlc) and was not examined further. The extract from incubation medium was chromatographed on silica gel using a benzene to ether solvent gradient (steps of 10% ether). This provided 20 (0.06 g, 6.7%), identified by comparison with an authentic sample; thioxanthone-10,10-dioxide, 43 (6 mg, 0.6%), and thioxanthone-10-oxide, 44 (10 mg, 1%). these products were identified by comparison of tlc, mp, and spectral data of products obtained from incubation # 18.

Section B

Microbial Oxidation of Alkylbenzenes by *Mortierella isabellina* NRRL 1757

The growth of the fungus, the incubation procedure and the work-up procedure were followed as stated in the previous experimental section.

Incubation # 29

Ethylbenzene , 28a

Incubation of 28a (0.6 g) gave extracts from mycelium (0.6 g) and medium (0.3 g). The former extract showed (tlc and nmr) no metabolic product and was discarded. The latter extract was applied to the top of a silica gel column. The column was eluted with benzene-ether (increments of 5% ether) to afford in order of decreasing R_f, ethylbenzene, 28a (0.12 g, 12%) and 1-phenylethanol, 51a (0.1 g, 10% yield); ^{1}H NMR δ : 1.2 (3H,d,J=6Hz), 2.7 (1H,s), 4.75 (1H,q,J=6Hz) and 7.2 (5H,s); MS m/e (%): 122 (12,$M^{+\cdot}$), 120 (19), 105 (80), 104 (68), 91 (48), 79 (45), 78 (57), 77 (100)

Incubation of ethylbenzene 28a with M. isabellina was repeated under the same conditions as described before but at different concentration levels.

(i) Incubation of 28a (0.5 g, 125 mg/flask) provided extracts from medium (0.2 g) and mycelium (0.25 g). TLC analysis of latter extract revealed the presence of starting material plus some endogenous fungal products. The former was chromatographed, as described above, and yielded 1-phenylethanol, 51a (80 mg, 16%).

(ii) Incubation of 28a (0.5 g, 50 mg/flask) gave extracts from mycelium (0.4g) and medium (0.18 g). The former indicated the presence

of starting substrate and some non polar oxidation product. The crude extract from incubation medium revealed (nmr) the presence of metabolic product. The column chromatography of this extract, as described above, gave very small amount of product which was not sufficient for spectral analysis and further investigations.

Incubation # 30

p-Ethyltoluene , 28b

Incubation of 28b (1.0 g) provided extracts from the medium (0.17 g) and mycelium (0.4 g). The latter contained endogenous fungal material and 28b as the only readily detectable compound. The former extract was chromatographed on silica gel using benzene-ether gradient elution (steps of 5% ether) and afforded 28b (0.07 g); 1-(p-methylphenyl)ethanol, 51b (38 mg, 3.8%); ^{1}H NMR δ : 1.43 (3H,d,J=6Hz), 2.05 (1H,s), 2.3 (3H,s), 4.8 (1H,q,J=6Hz), and 7.15 (4H,m); MS m/e (%): 136 (48,$M^{+\cdot}$), 121 (54), 118 (22), 117 (44), 107 (21), 105 (100), 93 (36), 77 (36); and 2-(p-methylphenyl)ethanol, 52a (10 mg, 0.1%); ^{1}H NMR δ :2.35 (3H,s), 2.8 (2H,t,J=6Hz), 3.8 (2H,t,J=6Hz), and 7.05 (4H,s); MS m/e (%): 136 (22,$M^{+\cdot}$), 118 (27), 105 (100), 103 (11), 91 (28), 79 (14), 77 (18)

Incubation # 31

p-Diethylbenzene , 28c

Incubation of 28c (1.0 g) gave extracts from mycelium (0.35 g) and medium (0.32 g). The former contained 28c and some natural fungal products (tlc) and was not examined further. The latter was applied to

a column of silica gel in benzene. The column was eluted with benzene-ether (increments of 5% ether) to give in order of elution, 28c (0.11 g, 11%), identical in all respects with starting material; 1-(p-ethylphenyl)ethanol, 51c (27 mg, 2.7%); ^{1}H NMR δ: 1.2 (3H,t), 1.35 (3H,d), 2.58 (2H,q), 3.45 (1H,s), 4.7 (1H,q), and 6.98 (4H,s); MS m/e (%): 150 (15,$M^{+\cdot}$), 135 (62), 132 (42), 117 (100), 107 (14), 105 (32), 79 (83), 77 (43); and 1-(p-acetylphenyl)ethanol, 53 (6 mg, 0.6%); ^{1}H NMR δ: 1.5 (3H,d,J=7Hz), 4.93 (1H,q,J=7Hz), and 7.38, 7.88 (4H,AB quartet,J=8Hz); MS m/s (%): 164 (5,$M^{+\cdot}$), 149 (60), 131 (26), 121 (66), 103 (25), 91 (11), 77 (26), 43 (100). A total of 0.03 g more polar fractions were also eluted which could not be identified, therefore not investigated further.

Incubation # 32

p-Methoxyethylbenzene , 28d

Incubation of 28d (1.0 g) provided extracts from the medium (0.29 g) and mycelium (0.41 g). The latter contained starting material as the only readily detectable compound (tlc) and was not examined further. The extract from incubation medium was chromatographed on siica gel using a benzene to ether solvent gradient (steps of 5% ether). This provided in order of elution, p-methoxyethylbenzene (0.05 g, 5%); 1-(p-methoxyphenyl)ethanol, 51d (0.1 g, 10%); ^{1}H NMR δ: 1.18 (3H,d,J=7Hz), 3.68 (3H,s), 4.6 (1H,q,J=7Hz), and 6.68, 7.1 (4H,AB quartet,J=9Hz); MS m/e (%): 152 (26,$M^{+\cdot}$), 137 (100), 134 (70), 121 (15), 119 (34), 109 (44), 91 (45), 77 (36). This incubation also gave

traces of another hydroxylation metabolite (nmr), 2-(p-methoxyphenyl)ethanol, 52b which could not be isolated in pure form and enough quantity for further investigations.

Incubation # 33

p-Fluoroethylbenzene , 28e

Incubation of 28e (1.0 g) gave extracts from the mycelium (0.5 g) and medium (0.19 g). the former contained 28e as the only readily detectable compound. there was no sign of any polar oxidation product and therefore it was not examined further. The latter extract was chromatographed on silica gel using benzene-ether gradient elution (steps of 5% ether). This provided, in order of decreasing R_f, p-fluoroethylbenzene (70 mg, 7%), identified by tlc and spectral comparison with a sample of authentic material; and a component (60 mg, 6%) with a lower R_f value than the starting substrate. The second component appeared (nmr) to be a mixture of 1-(p-fluorophenyl)ethanol, 51e (87%) and 2-(p-fluorophenyl)ethanol, 52c (13%). These isomers could not be separated by column chromatography, therefore the mixture was analysed qualitatively and used for further investigations. the ^1H NMR included signals at δ : 1.58 (3H,dJ=6Hz), 2.83 (2H,t,J=6Hz), 3.75 (2H,d,J=6Hz), 4.8 (1H,q,J=6Hz), and 7.13, 7.33 (4H,AB quatret,J=8Hz). The mass spectrum showed the following fragmentation pattern m/e (%): 140 (15,$M^{+\cdot}$), 122 (27), 117 (12), 109 (100), 105 (19), 96 (10), 91 (22), 77 (25)

Incubation # 34

p-Chloroethylbenzene , 28f

Incubation of 28f (1.0 g) gave extracts from medium (0.17 g) and mycelium (0.6 g). The latter contained 28f as the only readily detectable compound (tlc and nmr). The former extract gave a single spot on tlc with R_f lower than the starting material. This appeared to be a mixture of 1-(p-chlorophenyl)ethanol, 51f (81%) and 2-(p-chlorophenyl)ethanol, 52d (19%). These isomers could not be isolated in pure form by column chromatography. Thus the mixture was analysed qualitatively and used for further investigations. The ^1H NMR of the mixture included peaks at δ : 1.4 (3H,d,J=6Hz), 2.38 (1H,br,s), 2.75 (2H,t,J=6Hz), 4.8 (1H,q,J=6Hz), and 7.18 (4H,s). The mass spectrum of the mixture showed the following fragmentation pattern, m/e (%): 158 (5), 156 (17), 143 (19), 141 (64), 125 (29), 91 (17), 77 (100)

Incubation # 35

p-Cyanoethylbenzene , 28g

Incubation of 28g (1.0 g) provided extracts from the medium (0.6 g) and mycelium (0.59 g). The latter contained endogenous fungal material, plus 28g as the only readily detectable compound (tlc) and was not examined further. The extract from the incubation medium gave a single spot on tlc with R_f lower than starting material and was therefore used for further investigations without any purification. This compound was characterized as 1-(p-cyanophenyl)ethanol, 51g (0.6 g, 60%); ^1H NMR δ : 1.45 (3H,d,J=7Hz), 3.73 (1H,s), 4.88

(1H,q,J=6Hz), and 7.48 (4H,s); MS m/e (%): 147 (9,$M^{+\cdot}$), 132 (100), 129 (68), 128 (21), 116 (45), 104 (87), 102 (47), 77 (40)

Incubation # 36

p-Ethylaniline , 28h

Incubation of 28h (1.0 g) provided extracts from mycelium (0.49 g) and medium (0.53 g). The former contained 28h as the only readily detectable compound (tlc). The latter extract was chromatographed on silica gel by stepwise elution from benzene to ether (steps of 5% ether) to give p-ethylaniline (0.28 g, 28%), and p-ethylacetanilide, 28i (0.212 g, 21%), identified by mp, tlc, and spectral comparison with an authentic sample, prepared as described in a previous experimental section.

Incubation # 37

p-Ethylacetanilide , 28i

Incubation of 28i (1.0 g) gave extracts from medium (0.15 g) and mycelium (0.4 g). Thin layer chromatography of both extracts indicated the absence of any transformation products. The ^{1}H NMR of these extracts was identical with starting substrate.

Incubation # 38

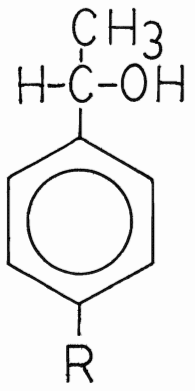
p-Ethylphenol , 28j

Incubation of 28j (1.0 g) provided extracts from mycelium (0.3 g) and medium (0.76 g). Both the mycelium and medium extract appeared (tlc) to be unmetabolized substrate. Further analysis by ^{1}H NMR showed that only starting material was present.

Incubation # 39

n-Propylbenzene , 29

Incubation of 29 (1.0 g) gave extracts from medium (0.64 g) and mycelium (0.4 g). TLC analysis of latter extract showed that it contained only 29 . The former extract was chromatographed on silica gel using benzene-ether solvent gradient elution (increments of 5% ether) and afforded 29 (0.09 g, 9%) and 1-phenylpropanol, 54 (0.45 g, 45% yield); ^{1}H NMR δ: 0.73 (3H,t,J=8Hz), 1.63 (2H,m), 3.13 (1H,s), 4.38 (1H,t,J=7 Hz), and 7.15 (5H,s); MS m/e (%): 136 (7,$M^{+\cdot}$), 118 (46), 115 (21), 107 (100), 105 (81), 91 (60), 79 (58), 77 (64).



51

a; R = H

b; R = CH_3

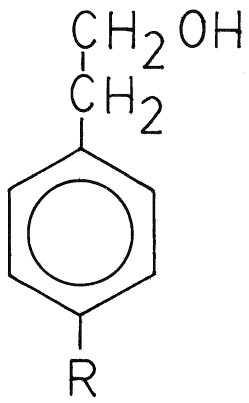
c; R = C_2H_5

d; R = OCH_3

e; R = F

f; R = Cl

g; R = CN



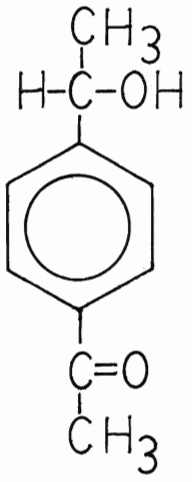
52

a; R = CH_3

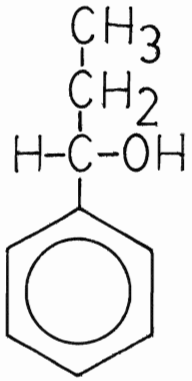
b; R = OCH_3

c; R = F

d; R = Cl



53



54

Section C

Microbial Reduction of Ketones by *Mortierella isabellina* NRRL 1757

All incubations were performed by standard procedure as previously described. The only difference was that the rubber stoppers were used in place of sponge plugs during incubation of fungus with substrate. Work-up method was identical to that described before for other cultures.

Incubation # 40

Acetophenone , 33a

Incubation of 35a (0.50 g) gave extracts from medium (0.48 g) and mycelium (0.2 g). The latter extract contained some endogenous fungal material, plus 33a as the only readily detectable compound (tlc) and was not examined further. The medium extract gave only one spot on tlc with R_f identical to that of 51a , and was therefore characterized without further purification. The compound was identified as 1-phenylethanol, 51a (0.435 g, 87% yield) by comparison of spectral data of a genuine sample obtained from incubation # 29.

Incubation # 41

p-Ethylacetophenone , 33c

Incubation of 33c (1.0 g) provided extracts from mycelium (0.31 g) and medium (0.59 g). TLC analysis of former extract revealed that it contained 35c as the only readily detectable compound. The latter was applied to the top of a column of silica gel. The column was eluted with benzene-ether (increments of 5% ether) to give in order of elution, 33c (0.2 g), identical in all respects with starting material, and

1-(p-ethylphenyl)ethanol, 51c (0.07 g, 7% yield), identified by tlc and spectral comparison with an authentic sample obtained from incubation # 31.

Incubation # 42

α,α,α-Trifluoroacetophenone , 36

Incubation of 36 (0.50 g) gave extracts from medium (0.43 g) and mycelium (0.2 g). The latter contained natural fungal products in addition to 36 as the only detectable compound (tlc). The former extract appeared (tlc) to be a pure compound. Thus it was identified as 1-phenyl,2,2,2-trifluoroethanol, 34 (0.43 g, 86%). Its ^{1}H NMR spectrum showed peaks at δ : 4.0 (1H,br,s), 4.68 (1H,q), and 7.3 (5H,s). The ^{19}F NMR of the product included a signal at δ : -77.4 (3F,d,J=6.6Hz). The mass spectrum showed the following fragmentation pattern, m/e (%): 176 (26,$M^{+\cdot}$), 140 (29), 127 (17), 109 (13), 107 (100), 105 (37), 79 (64), 77 (72).

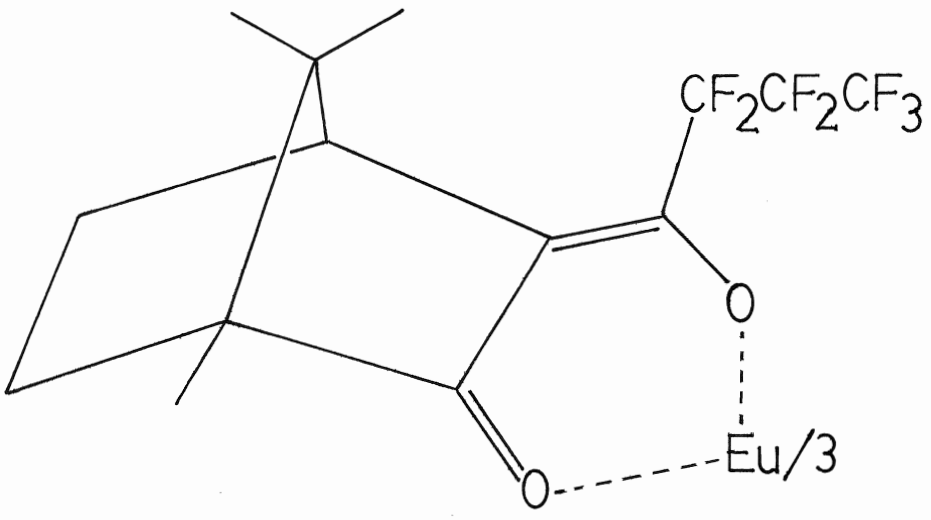
PART THREE

Determination of Enantiomeric Excess

The enantiomeric enrichments were determined by the ^{1}H NMR chiral shift reagent method. Shift reagent, Eu(hfc)$_3$ (Fig. 33) and the chiral compound in study were dissolved in 0.5 mL of reagent grade carbon tetrachloride. The molar ratio of shift reagent to chiral compound was 0.2 - 0.3. In some cases more shift reagent was required in order to increase the enantiomeric shift difference ($\Delta\Delta\delta$) of methyl and ortho protons. The addition of shift reagent caused the downfield shift of benzylic, methyl and ortho protons. The peak of the benzylic and methyl protons which appeared as a quartet and doublet without shift reagent, split into a multiplet and triplet (composed of two overlaping quartets and doublets, respectively, from two enantiomers). By irradiating at multiplet, the triplet collapsed to a resolved ($\Delta\Delta\delta$Ca. 0.05 - 0.2 ppm) doublet. The relative peak areas were integrated and the ratio of the enantiomers was calculated from the average of fifteen to twenty integrations. In addition, the ortho protons also appeared as a triplet (overlapping doublets) in the presence of Eu(hfc)$_3$ while the meta protons were still a doublet. By decoupling at the doublet signal (meta protons), the triplet (ortho protons) collasped to two signals of different peak areas (see appendix II). The integration of these two peaks was also carried out fifteen to twenty times and these values were averaged to obtain the ratio of two enantiomers. The values obtained from methyl and ortho protons were averaged (in most cases) to determine the enantiomeric excess.

The results are presented in Table 8.

Figure 33. The structure of chiral shift reagent



$Eu(hfc)_3$ = tris[3-(heptafluoropropylhydroxymethylene)-d-camphorato]-europium(III)

Table 8. Optical purities of products

Incubation #	Product	Ratio of Enantiomers %	Enantiomeric Excess %
29.	51a	67 : 33[a]	34
29(i).	51a	69 : 31	38
30.	51b	62 : 38	24
31.	51c	68 : 32	36
32.	51d	66 : 34	32
33.	51e	56 : 44	12
34.	51f	67 : 33	34
35.	51g	69 : 31	38
39.	54	b	--
40.	51a	92 : 08	84
41.	51c	100 : 00	100
42.	34	c	--

a Measured by peak height because peaks were too close and integration was not useful.

b Decoupling of β protons was not successful.

c Not determined due to nonavailability of heterodecoupling ie $^{19}F\{^{1}H\}$ or vice versa.

PART FOUR

Determination of Specific Rotations

Optical rotations were measured in 95% ethanol using 1 mL sample cell at 21°C. The specific rotations were calculated using the following equation.

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{C\ l}$$

Where,

α is the observed rotation in degrees.

C is the concentration in g/100 mL of solution.

l is the length of the sample cell in decimeters.

t is the temperature in °C and

λ is the wave legnth.

The results are listed in Table 9.

The values of measured specific rotations so obtained were used where possible to check the enantiomeric enrichments. For these calculations, the specific rotations of pure enantiomers were used.

Table 9. Specific rotations of products and their absolute configurations

Incubation No.	Product	[∝] in 95% EtOH	[∝] for the pure enantiomer (lit.)	Concentration [g/100 mL of solution]	Configuration[88,98,176]
16.	42	-14.04	--	2.77	--
19.	49	-6.67	--	0.27	--
29.	51a	+12.65	43.9[173]	0.83	R
29(i).	51a	+11.44	43.9[173]	1.25	R
30.	51b	+3.00	--	0.40	R
31.	51c	+2.00	--	0.25	R
31.	53	+3.42	--	0.38	--
32.	51d	-9.41	45.2[174]	1.02	S
33.	51e	+38.68	--	0.91	R
34.	51f	-4.04	--	4.43	S
35.	51g	+10.33	--	8.56	R
39.	54	+12.98	55.54[175]	7.07	--
40.	51a	-5.41	43.9[173]	3.57	S
41.	51c	+73.93	--	0.56	R
42.	34	-2.65	--	7.32	--

R E S U L T S

PART ONE

Biotransformation of Polycylic Aromatic Compounds by Fungi

Table 10. Summary of results

Incubation No.	Substrate	Fungus	Compound/s isolated (%)
1.	Dibenzothiophene, 1	C. elegans	Dibenzothiophene-9,9-dioxide, 37 (2.8) Dibenzothiophene-9-oxide, 38 (0.5)
2.	Dibenzopyrrole, 2	C. elegans	2-Hydroxydibenzopyrrole, 39 (1)
3.	Fluorene, 3	C. elegans	Starting material, 3 (40)
4.	9-Fluorenone, 4	C. elegans	9-Hydroxyfluorene, 40 (1.4)
5.	Acridine, 5	C. elegans	Structure not determined
6.	Phenanthridine, 6	C. elegans	Starting material, 6 (88)
7.	1:10-Phenanthroline, 7	C. elegans	Starting material, 7 (85)
8.	Benz[a]anthracene, 8	C. elegans	Starting material, 8 (76)
9.	Benz[c]acridine, 9	C. elegans	Starting material, 9 (79)
10.	Triphenylphosphine, 12	C. elegans	Triphenylphosphine oxide, 41 (18)

Table 10, continued (p. 2)

Incubation No.	Substrate	Fungus	Compound/s isolated (%)
11.	Dibenzo-1,4-dioxin, 13	C. elegans	Starting material, 13 (65)
12.	5H-Dibenzo[a,d]cyclohepten-5-one, 14	C. elegans	Starting material, 14 (100)
13.	Dibenzosuberone, 15	C. elegans	Starting material, 16 (100)
14.	Thianaphthene, 16	C. elegans	Starting material, 16 (64)
15.	Alpha-terthienyl, 17	C. elegans	Starting material, 17 (69)
16.	Thiochroman-4-one, 18	C. elegans	Thiochromanone-1-oxide, 42 (16)
17.	2-Phenylthiochroman-4-one, 19	C. elegans	Starting material, 19 (57)
18.	Thioxanthan-9-one, 20	C. elegans	Thioxanthone-10,10-dioxide, 43 (2) Thioxanthone-10-oxide, 44 (0.5)
19.	1-Methylthioxanthone, 21	C. elegans	1-Methylthioxanthone-10,10-dioxide, 45 (2.5) 1-Hydroxymethylthioxanthone-10-oxide, 49 (7)

Table 10 continued (p. 3)

Incubation No.	Substrate	Fungus	Compound/s isolated (%)
20.	2-Methylthioxanthone, 22	C. elegans	2-Methylthioxanthone-10,10-dioxide, 46 (5)
21.	3-Methylthioxanthone, 23	C. elegans	3-Methylthioxanthone-10,10-dioxide, 47 (4.4) 3-Hydroxymethylthioxanthone, 50 (6)
22.	4-Methylthioxanthone, 24	C. elegans	4-Methylthioxanthone-10,10-dioxide, 48 (10)
23.	Dibenzothiophene, 1	Rhizopus arrhizus	Dibenzothiophene-9,9-dioxide 37 (0.6) Dibenzothiophene-9-oxide 38 (0.2)
24.	Canadine, 25	R. arrhizus	Starting material, 25 (75)
25.	Tetrahydropalmatine, 26	R. arrhizus	Starting material, 25 (82.5)
26.	Corydaline, 27	R. arrhizus	Starting material, 27 (77.5)

Table 10, continued (p. 4)

Incubation No.	Substrate	Fungus	Compound/s isolated (%)
27.	Dibenzothiophene, 1	Mortierella isabellina	Dibenzothiophene-9,9-dioxide, 37 (5) Dibenzothiophene-9-oxide, 38 (1)
28.	Thioxanthan-9-one, 20	Mortierella isabellina	Thioxanthone-10,10-dioxide, 43 (0.6) Thioxanthone-10-oxide, 44 (1)

PART TWO

Microbial Oxidation of Alkylbenzenes by *Mortierella isabellina* NRRL 1757

Table 11. Summary of results

Incubation No.	Substrate	Compounds isolated (%)	Enantiomeric excess %	
			NMR	Rotation
29.	Ethylbenzene, 28a	1-phenylethanol, 51a (10)	34	28.82
29(i).	Ethylbenzene, 28a	1-phenylethanol, 51a (16)	38	26.10
30.	p-Ethyltoluene, 28b	1-(p-methylphenyl)ethanol 51b (3.8)	24	--
		2-(p-methylphenyl)ethanol 52a (0.1)	--	--
31.	p-Diethylbenzene, 28c	1-(p-ethylphenyl)ethanol 51c (2.7)	36	--
		1-(p-acetylphenyl)ethanol 53 (0.6)	--	--
32.	p-Methoxyethylbenzene, 28d	1-(p-methoxyphenyl)ethanol 51d (10)	32	20.82
		2-(p-methoxyphenyl)ethanol 52b (*)	--	--

Table 11, continued (p. 2)

Incubation No.	Substrate	Compound/s isolated	Enantiomeric excess %	
			NMR	Rotation
33.	p-Fluoroethylbenzene, 28e	1-(p-fluorophenyl)ethanol 51e	12	--
		2-(p-fluorophenyl)ethanol 52c (**)	--	--
34.	p-Chloroethylbenzene, 28f	1-(p-chlorophenyl)ethanol 51f	34	--
		2-(p-chlorophenyl)ethanol 52d (**)	--	--
35.	p-Cyanoethylbenzene, 28g	1-(p-cyanophenyl)ethanol 51g (60)	38	--
36.	p-Ethylaniline, 28h	p-ethylacetanilide, 28i (21)	--	--
37.	p-Ethylacetanilide, 28i	starting material, 28i (55)	--	--
38.	p-Ethylphenol, 28j	starting material, 28g (100)	--	--
39.	n-Propylbenzene, 29	1-phenylpropanol, 54 (45)	--	23.27

* Product detected (tlc) and qualitatively analysed (nmr) but not isolated in pure form.

** Two isomers were not isolated separately.

PART THREE

Microbial Reduction of Ketones by *Mortierella isabellina* NRRL 1757

Table 12. Summary of results

Incubation No.	Substrate	Compound/s isolated (%)	Enantiomeric excess %	
			NMR	Rotation
40	Acetophenone, 33a	1-phenylethanol, 51a (87)	84	12.43
41.	p-Ethylacetophenone, 33c	1-(p-ethylphenyl)ethanol 51c (7)	100	--
42.	α,α,α-Trifluoroacetophenone, 36	1-phenyl-2,2,2-trifluoro-ethanol, 34 (87)	--	--

D I S C U S S I O N

PART ONE

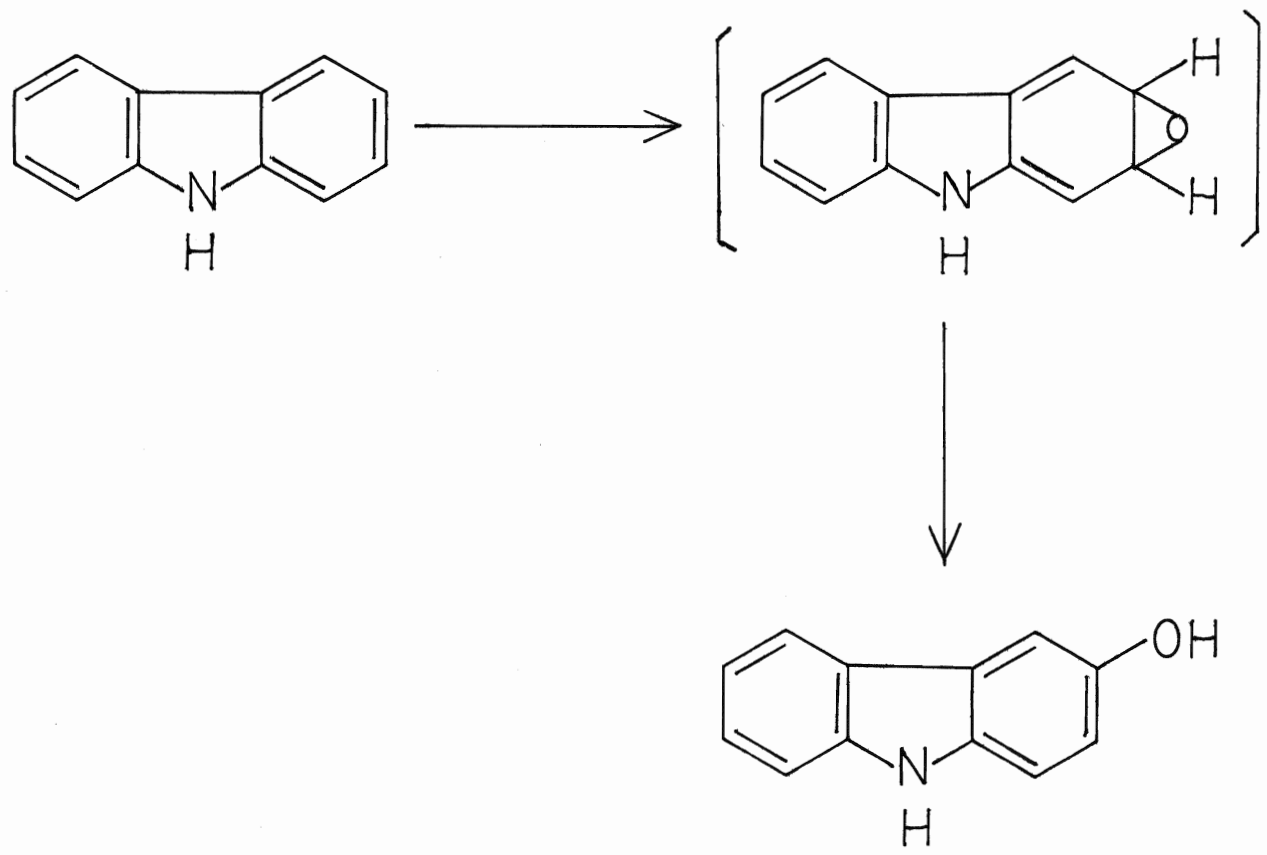
Biotransformation of Polycylic Aromatic Compounds by Fungi

Many chemical carcinogens are now known to be activated and transformed *in vivo* into highly active metabolites.[177,178] Although the relevant metabolism for PACs has not received a great deal of recent attention, however polycyclic aromatic hydrocarbons have been the subject of intensive scrutiny.[10,11,56,66,179,180,181]

Of the fungi studied, *C. elegans* has been the subject of much recent study and is known to catalyse various reactions by cytochrome P-450 dependent monooxygenases.[64,179] This organism demonstrated a high degree of parallelism to liver microsomal monooxygenase systems.[11,56,64,67]

Of the aza heteroaromatics studied, only dibenzopyrrole, 2 gave a cleanly isolable product, 2-hydroxydibenzopyrrole, 39 . Fungal oxidation of dibenzopyrrole at C-2 parallels the reported metabolism of dibenzofuran to 2-hydroxylated derivative by *C. elegans.*[57] The reaction sequence shown in Figure 34 is proposed for the metabolism of dibenzopyrrole. No evidence for the formation of arene oxide was obtained; it is proposed by analogy with the proposed pathway for fungal hydroxylation of dibenzofuran at C-2, and also by the fact that most arene oxides undergo spontaneous isomerization to form phenols. Incubation in the presence of protein synthesis inhibitor, cycloheximide (100 μg/mL) did not show any significant inhibition of enzymic activity. It is known that cycloheximide inhibits the hydroxylation of progesterone by *Rhizopus nigricans*[158] when added at the

Figure 34. Proposed reaction sequence for the metabolism of Dibenzopyrrole by C. elegans



concentration of 100 μg/mL, proving the inducible nature of progesterone hydroxylating enzymes. However the results obtained in this study do not necessarily mean that enzyme present in C. elegans which is responsible for hydroxylation of dibenzopyrrole is noninducible. The difference between the two cases in respect of inducibility could be explained by the fact that two different fungi are involved.

Acridine, 5 gave traces of an oxidized product which was partially characterized (see experimental) as a ring hydroxylated product but could not be isolated in sufficient quantity for complete characterization. The induction of enzyme activity was unsuccessfully attempted by the addition of substrate during the growth period of the fungi. The corresponding polynuclear hydrocarbon, anthracene, has been recently reported[181] to be oxidized by C. elegans to transdihydrodiols.

Incubation of phenanthridine, 6 with C. elegans showed no sign of any metabolic product. In contrast, Benson and coworkers[44] observed the metabolism of this compound by rat lung and liver microsomes to several more polar metabolites. The pattern of metabolites produced by lung and liver is qualitatively similar. Phenanthrene, the analogous hydrocarbon has been reported to be metabolized by bacteria[182] and fungi[181] to corresponding cis and trans dihydrodiols, respectively.

1:10 Phenthanroline, 7 and benz[c]acridine, 9 were recovered unchanged after incubation with C. elegans . However, Jacob

et al [42] have recently reported the metabolic activation of benz[c]acridine by rat liver and lung microsomes.

A wide variety of thia heteroaromatics were incubated with different fungi. The results are presented in Table 10. Dibenzothiophene, 1 gave two products on incubation with all the three fungi studied. The products were identified as the corresponding sulphone, 37 and sulphoxide, 38 . No ring hydroxylated products were detected. The differences in yield of two metabolites with different fungi may be due to many factors. For instance, the optimum conditions used for incubations may not represent the optimum conditions for dibenzothiophene biotransformation. Also the yield of metabolites depends upon factors such as supply of nutrients, incubation time, temperature and pH.

Although earlier workers[114,183,184] have reported that sulphides are metabolized to the corresponding sulphoxides and sulphones by a range of fungi, to our knowledge the present results constitute the first example of fungal oxidation of sulphur in a heteroaromatic ring. Since the metabolic route is unique and the products formed to a lesser extent by fungi, certain control experiments are required to establish the dependence of this pathway on enzymic systems. The incubation of dibenzothiophene was performed with an autoclaved culture of C. elegans ; in these conditions no product was detected, confirming that the products were indeed derived from an enzymic process. The formation of products by C. elegans was not inhibited by cycloheximide which

suggests that enzyme is non-inducible. To obtain more convincing results about the inducible nature of enzyme, it is necessary to carry out these incubations in the presence of classical cytochrome P-450 inhibitors, metyrapone and SKF 525-A. The corresponding bicyclic compound thianaphthene, 16 was not metabolized by C. elegans . Previous reports of the bacterial metabolism of dibenzothiophene indicated the formation of ring oxidized products.[185,186] This contrast between the metabolic pathways of bacteria and fungi may be significant in view of the use of fungi as models for mammalian metabolism. However, it would be of interest to see if mammalian microsomal systems show similar metabolic routes to that observed with fungal systems.

Thioxanthan-9-one, 20 was found to be oxidized at sulphur by both C. elegans and M. isabellina . Corresponding sulphone, 53 and sulphoxide, 44 were obtained in both the cases, but the former predominating in the case of C. elegans while the latter was the major product in the case of M. isabellina. Interestingly, the corresponding bicyclic compound, thiochroman-4-one, 18 was stereoselectively metabolized to thiochromanone-1-oxide, 42 by C. elegans . This observation is consistent with the findings of Holland et al [102] who reported the metabolism of organic sulphides by M. isabellina to optically active sulphoxides. Auret and coworkers[187] reported similar results with Aspergillus niger .

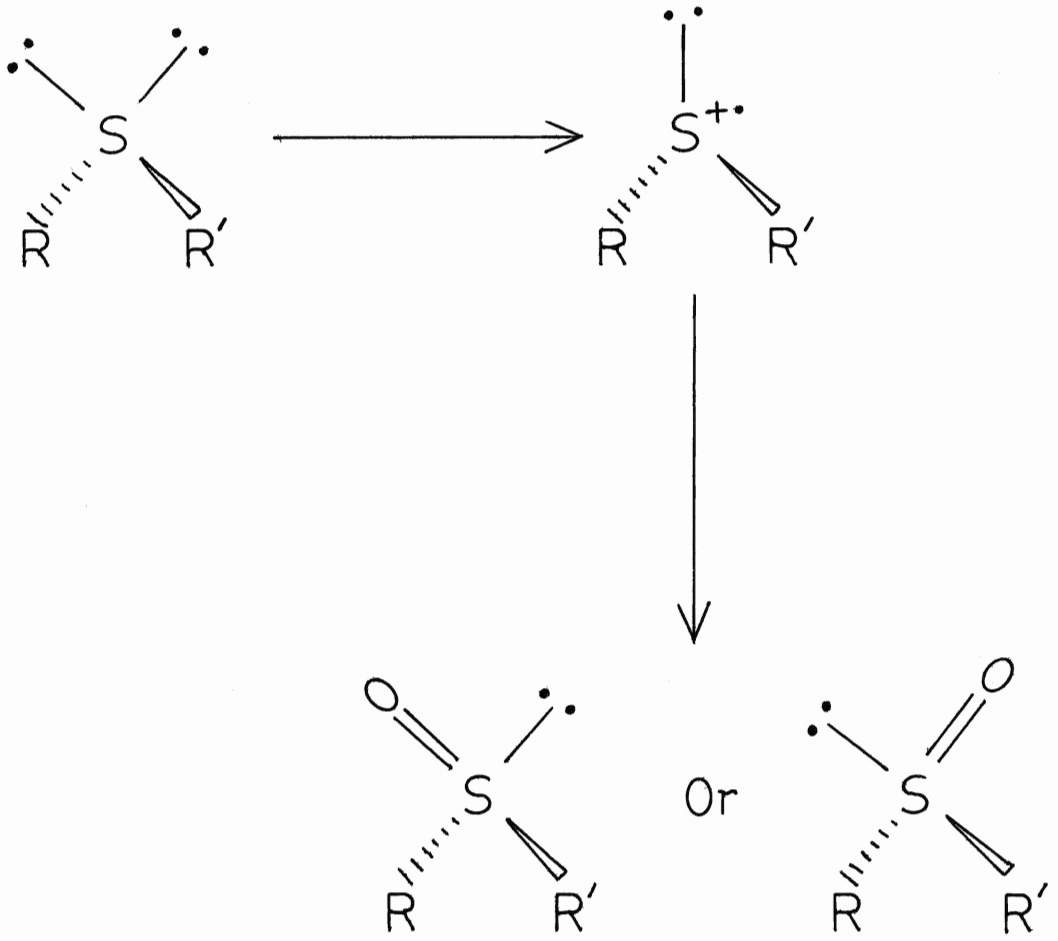
2-Phenylthiochromanone, 19 was not metabolized at all. This may

be explained on the basis of fact that carbon adjacent to sulphur possesses a bulky substituent which might have sterically hindered the attack of oxidizing species on the sulphur atom.

Previous work[114,149,150] with sulphides has indicated that oxidation at sulphur is a stepwise process; the intermediate has been formulated as the radical cation (Figs. 30 and 35). The intermediate will not possess the regular tetrahedral geometry characteristic of sulphides and can invert at room temperature. Therefore, it can be proposed that the optical purity and absolute stereochemistry of the product are dependent on the extent of stereospecificity of binding in active site and the stability of radical cation intermediate.

All the four methyl substituted thioxanthones, 21 , 22 , 23 and 24 were actively converted to corresponding sulphones 45 , 46 , 47 and 48 by C. elegans. In addition this fungus also metabolized 1-methylthioxanthone, 21 to optically active 1-hydroxymethylthioxanthone-10-oxide, 49 . In contrast the 3-methylthioxanthone, 23 was converted to 3-hydroxymethylthioxanthone, 50 . It is possible that the enzyme that catalyses sulphoxygenation also carries out methyl group hydroxylation. The presence and position of the methyl substituent in the aromatic ring affected the regioselectivity of the enzyme and thus changed the metabolite pattern. Thus, the results indicate that the fungal enzymes show considerable variation in the regio-selective metabolism of methylthioxanthones. It would be necessary to perform the incubation of 3-methylthioxanthone, 23

Figure 35. Mechanism of enzymic sulphide oxidation



under an $^{18}O_2$ atmosphere to determine whether hydroxylation and sulphoxygenation are both catalysed by a monooxygenase.

Recently Cerniglia and coworkers[179] have reported that the fungus C. elegans oxidises 4-methylbenz[a]anthracene primarily at the methyl group with further oxidation and enzymic hydration to form various dihydrodiols (Fig. 36). Incubation under $^{18}O_2$ atmosphere and subsequent mass spectral analysis of metabolites indicated that both methyl group and aromatic ring are hydroxylated by the biotransformation catalysed by cytochrome P-450 dependent monooxygenase. The same authors[180] have reported that 1- and 2-methylnaphthalenes are also hydroxylated by C. elegans , primarily at the methyl group (Fig. 37), and the metabolism is catalysed by a monooxygenase.

Incubation of fluorene, 3 and benz[a]anthracene, 8 showed no sign of biotransformation. In contrast, bacterial and mammalian metabolism of benz[a]anthracene has been reported by various groups.[50,188,189]

Of the polycyclic aromatic ketones studied, only 9-fluorenone, 4 was metabolized by C. elegans to 9-hydroxyfluorene, 40 . Reduction of keto group of fluorenone is interesting since it represents a common mammalian biotransformation.[74] Fungal reduction of carbonyl group of 4 to alcohol is quite similar to the reported conversion of fluorenone to fluorenol by liver microsomal preparations,[190] and likewise is similarly assumed to be catalysed by NADPH-cytochrome P-450 reductase and NADPH. This metabolism suggests close similarity between

Figure 36. Microbial metabolism of 4-methylbenz[a]anthracene by the fungus C. elegans

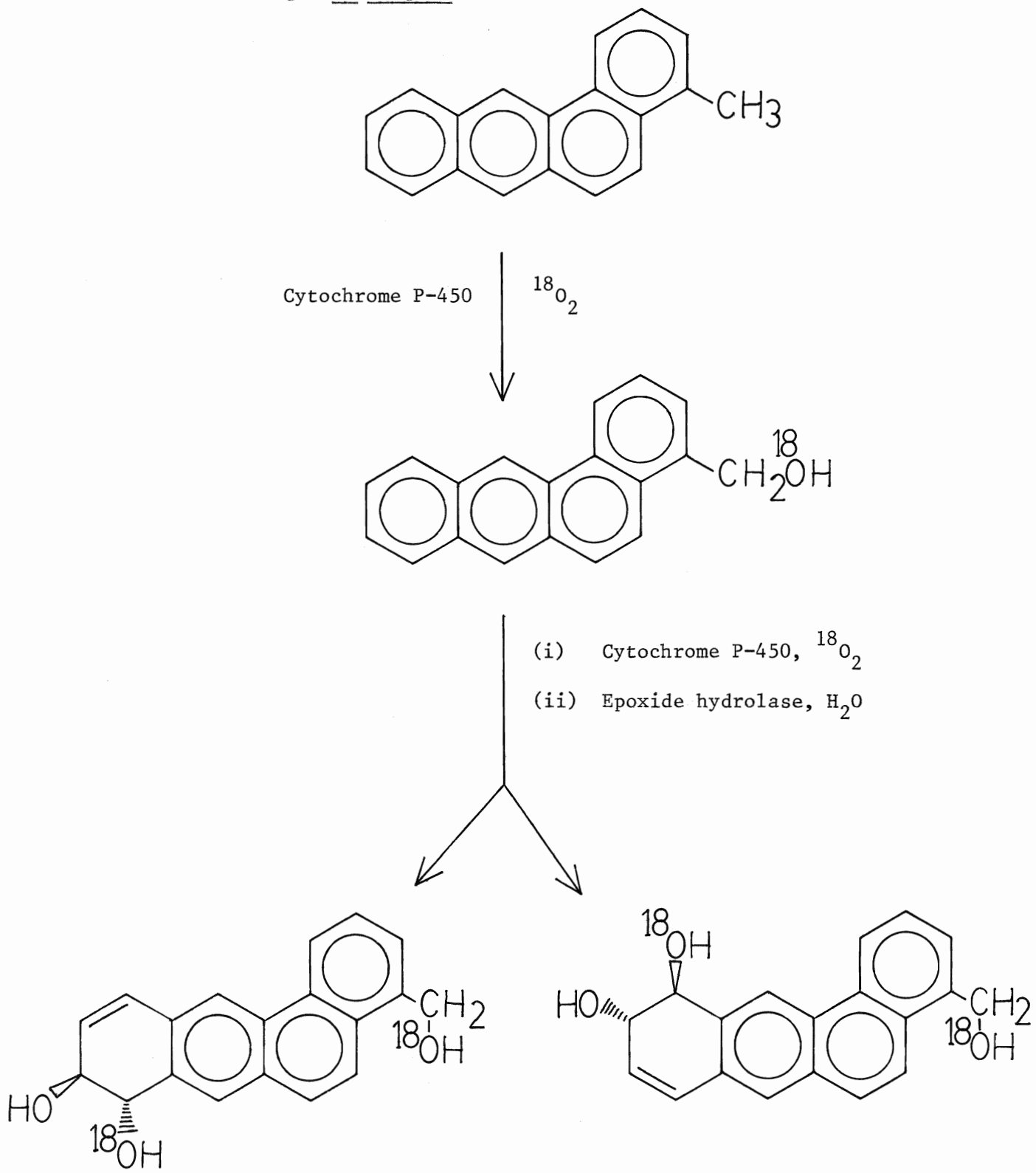
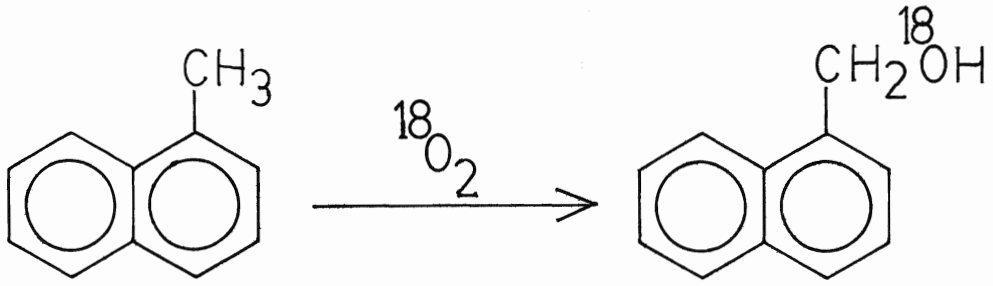
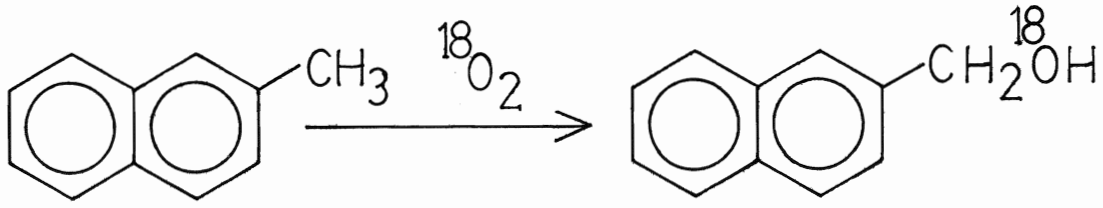


Figure 37. Transformation of 1- and 2-Methylnaphthalenes by C. elegans





the fungal and mammalian metabolism.

The only phosphorus containing compound, triphenylphosphine, 12 used in this study appeared to be principally converted to triphenylphosphine oxide, 41 by the fungi used. However, the control experiment carried out using an autoclaved culture of C. elegans revealed the formation of same product and up to the similar extent, thereby indicating the nonenzymatic formation of triphenylphosphine oxide, 41 .

The results obtained in this study demonstrate that C. elegans a typical soil fungus has the ability to oxidize many important environmental pollutants. Possible comparisons of fungal vs. mammalian transformations indicated that metabolites are identical. Thus, the possibility exists that further studies will lead to the identification of new products that have yet to be detected as mammalian metabolites. In addition, the relative importance of these reactions in the degradation of polycyclic aromatic compounds in natural ecosystems may be established.

Despite the variety of reactions and the diversity of the substrates which are metabolized, the fungus show in many cases regiospecificity and stereoselectivity. This may partly be contributed by multiple forms of cytochrome P-450 present in the fungi. It would be interesting to examine the effects of classical cytochrome P-450 inducers (e.g., phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls) on variable enzyme activity in this organism.

Although many metabolites were estimated to occur in low yields, it should be emphasized that it is usually possible to improve yields by varying a number of incubation parameters. Among the experimental conditions which may be changed for this purpose are: medium composition, time of substrate addition, time of incubation with substrate, the degree of aeration, and the use of inducers.

The incubations of many compounds with C. elegans and R. arrhizus failed to produce corresponding oxidation products. In all these cases, only starting material was recovered. There are three possibilities that may explain why problems were encountered with the possible metabolism. The first possibility is that the substrate was unable to cross the cell membrane. The second is that the substrate could not enter the active site of the enzyme and the third, that the substrate was toxic to the fungi or acted as an inhibitor to enzyme. Since most of the starting materials were recovered through extraction from fungal mycelia, however, the inactivity of the enzyme is not due to the inability of the substrate to enter the cell, but with enzyme itself. It may be assumed that apoenzyme was incapable to orient the substrate correctly for reaction. In dealing with the membrane bound enzymes these problems are not unexpected. The possibility that these substrates were lethal to the fungus, although not proven, is unlikely.

It seems probable that cytochrome P-450 is responsible for the oxidation of various substrates by C. elegans . This conclusion is based on the observation that in certain cases, there existed a stronger

parallel between the metabolites formed by cytochrome P-450 dependent systems described in the literature and the fungal metabolites observed in the present study. However, further studies are necessary on the mammalian microsomal metabolism of thia heteroaromatic compounds in order to obtain a valid comparison of results obtained in the present study and possible use of C. elegans as a microbial model of mammalian metabolism.

PART TWO

Microbial Oxidation of Alkylbenzenes by *Mortierella isabellina* NRRL 1757

In addition to environmental concerns, many fungi are also important for synthetic purposes in view of their broad substrate specificity and high regio and stereoselectivity. Therefore, fungus is a unique catalyst which can also be exploited by synthetic organic chemist to achieve his/her goals which may otherwise challenge his or her synthetic ingenuity and skill.

Holland and coworkers[102,114] observed that a series of alkyl aryl sulphides on incubation with Mortierella isabellina resulted in stereoselective transformation to the corresponding sulphoxides and the stereoselectivity of S oxidation is susceptible to electronic character of para substituent. They proposed that cytochrome P-450 dependent monooxygenase enzymes of the fungus were responsible for oxidation at sulphur. They also observed that the hydroxylation of ethylbenzene to 1-phenylethanol is also carried out by the same enzyme system.[191] On the basis of these observations, similar results were proposed for the α-hydroxylation of para-substituted ethylbenzenes by M. isabellina under similar experimental conditions. It is therefore the purpose of this study to investigate this hydroxylation with ethylbenzene and its para-substituted derivatives.

The fungus Mortierella isabellina NRRL 1747 hydroxylated para-substituted ethylbenzenes, 28a-g and propylbenzene, 29 to give the corresponding 1-phenylethanols, 51a-g and 1-phenylpropanol, 54, respectively, with varying yields and optical purities; this is

summarized in Table 11. In the case of p-methyl, 28a ; p-methoxy, 28d ; p-fluoro, 28e ; and p-chloroethylbenzene, 28f , the corresponding 2-phenylethanols 52a-d were also formed. Interestingly, p-diethylbenzene was also metabolized to 1-(p-acetylphenyl)ethanol, 53 .

p-Ethylaniline, 28h was actively converted to p-ethylacetanilide, 28i . No hydroxylation product was obtained. N-acetylation of aniline is interesting since this represents a common mammalian biotransformation observed particularly with aromatic amines,[192,193] This product is somewhat unexpected but by no means unique. It was demonstrated over a decade ago that a variety of fungi are capable of transforming aniline to acetanilide.[72] On the other hand, Ferris et al [66] reported that C. bainieri converts aniline to its 4-hydroxy derivative, a result which was confirmed by other researchers.[72]

p-Ethylacetanilide, 28i and p-ethylphenol, 28j were not metabolized by M. isabellina at all. In contrast, Smith and Rosazza[72] have shown that acetanilide is principally converted to 2-hydroxyacetanilide and aniline by A. ochraceous . Interestingly, four other fungi deacetylated the substrate forming aniline.[72] The inactivity of enzyme shown in the present study may be explained by steric problems, caused by the presence of ethyl moiety at para position, in the active site of enzyme system. In a recent study, McIntire and coworkers[98] have indicated the formation of 1-(p-hydroxyphenyl)ethanol from p-ethylphenol by P. putida and highly

purified p-cresol methylhydroxylase, an unusual flavocytochrome. In both the cases, an excess of (S)-(-) enantiomer was observed.

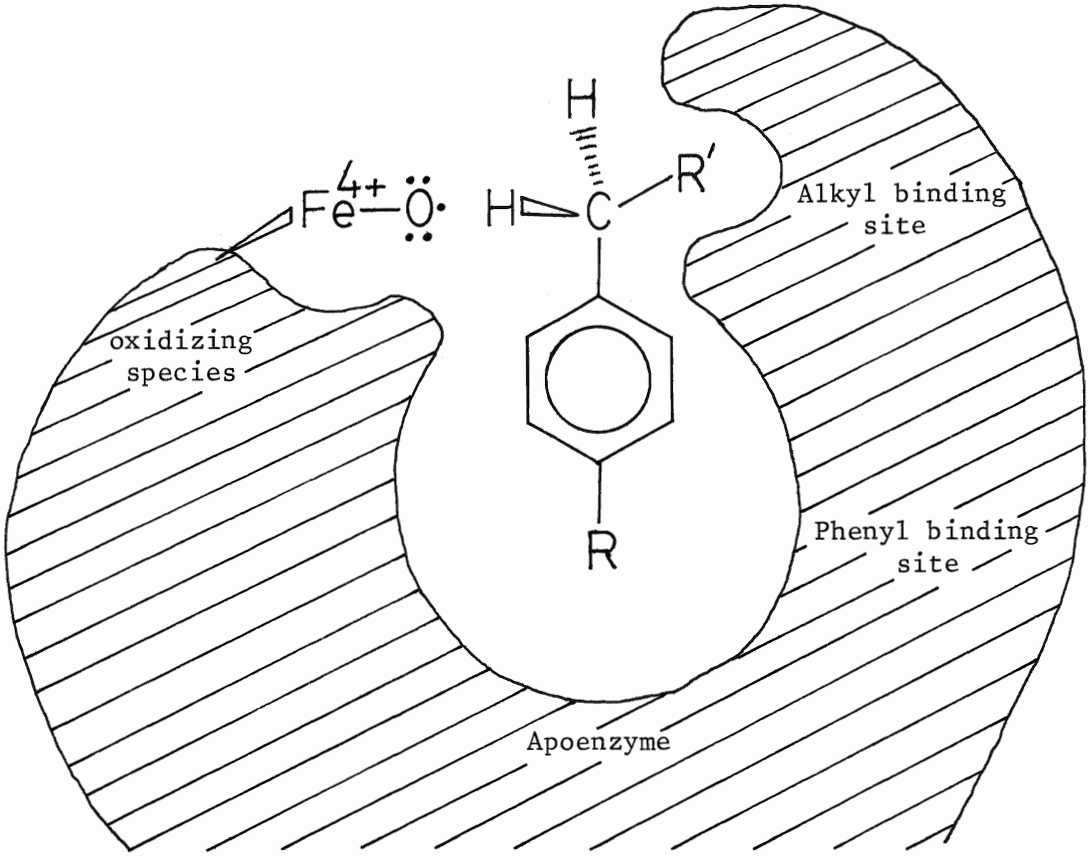
The results obtained in this study (Table 11) clearly indicate that major metabolic pathway involves α-hydroxylation of substrates, 28a-g providing optically active alcohols, 51a-g in all the cases.

To account for the formation of optically active alcohols, the enzyme system/s must be able to distinguish between the enantiotopic hydrogens at the prochiral carbon centre. In an enzymic system this can be easily achieved by providing binding sites which are different in size and shape and hence constituting an asymmetric environment in the active site of the enzyme allowing the substrate to bind, preferably in one way. The protein body, 'apoenzyme' is responsible for this stereospecific binding (Fig. 38). In the case shown in Figure 38 where stereospecific binding pockets for the substrate as well as fixed stereospecific position of oxidizing species relative to the substrate are assumed, only one enantiomer would be expected. Variation of R and R' should have no effect on optical purity whether the transformation proceeds in two steps or is concerted.

Since the hydroxylation of substrates provided a mixture of two enantiomers with one enantiomer in excess, the model of Figure 38 has to be modified.

An alternate model may involve a loose binding of ethyl group in the active site of enzyme. So if the reaction is a stepwise process and

Figure 38. Model for the binding of a para-substituted Alkylbenzene



a cation radical intermediate is formed, there is possibility that inversion of intermediate can occur whose extent would depend in part on its own stability. Since radicals are electron deficient, the nature of the para substituent R could have an electronic effect which stabilizes or destabilizes the radical intermediate, depending on whether the substituent is electron donating or withdrawing. This would allow inversion to occur more readily in the former and less readily in the latter case, raising the possibility that the optical purity of product should depend at least in part on the electronic character of para substituent.

On the other hand, if the metabolic pathway involves the concerted mechanism there should be no effect of para substituent as there is no possibility of inversion and reaction is entirely from one side.

A comparison of the enantiomeric excesses of alcohols obtained from isosteric substrates (Table 11) suggests that the ee is dependent on the electron density at prochiral carbon center, in some cases. Thus with substrates having substituents of similar steric size and different electronic nature such as 28d/28c (para OCH_3 vs. para C_2H_5), ee apparently increases from 32 to 36%. A similar effect is observed for 28b/28g (para CH_3 vs. para CN) where ee increases from 24 to 38%, suggesting that, for these substrates, an increase in electron withdrawal by the aromatic ring and the resultant destabilization of the cation radical intermediate causes an increase of enantiomeric excess. In contrast, comparison of 28a and 28e (para H vs. para F) showed a

substantial decrease in ee from 34 to 12%, while in principle there should be a significant increase in ee as fluorine is a good destabilizer of intermediate. The basis for this discrepancy is not clear, however, this inconsistent observation and small magnitude of increase in ee observed in previous two cases argue against the proposed hypothesis that stereoselectivity is susceptible to electronic effect of the substituent at the para position of the aromatic ring. The change of absolute stereochemistry observed in the case of electron donating and electron withdrawing substituents, OCH_3 and Cl, respectively, is not in agreement either with described model or with the proposed hypothesis discussed above. It should be pointed out, however, that comparison of optical purities of chiral alcohols with steric size of para substituents also showed no correlation.

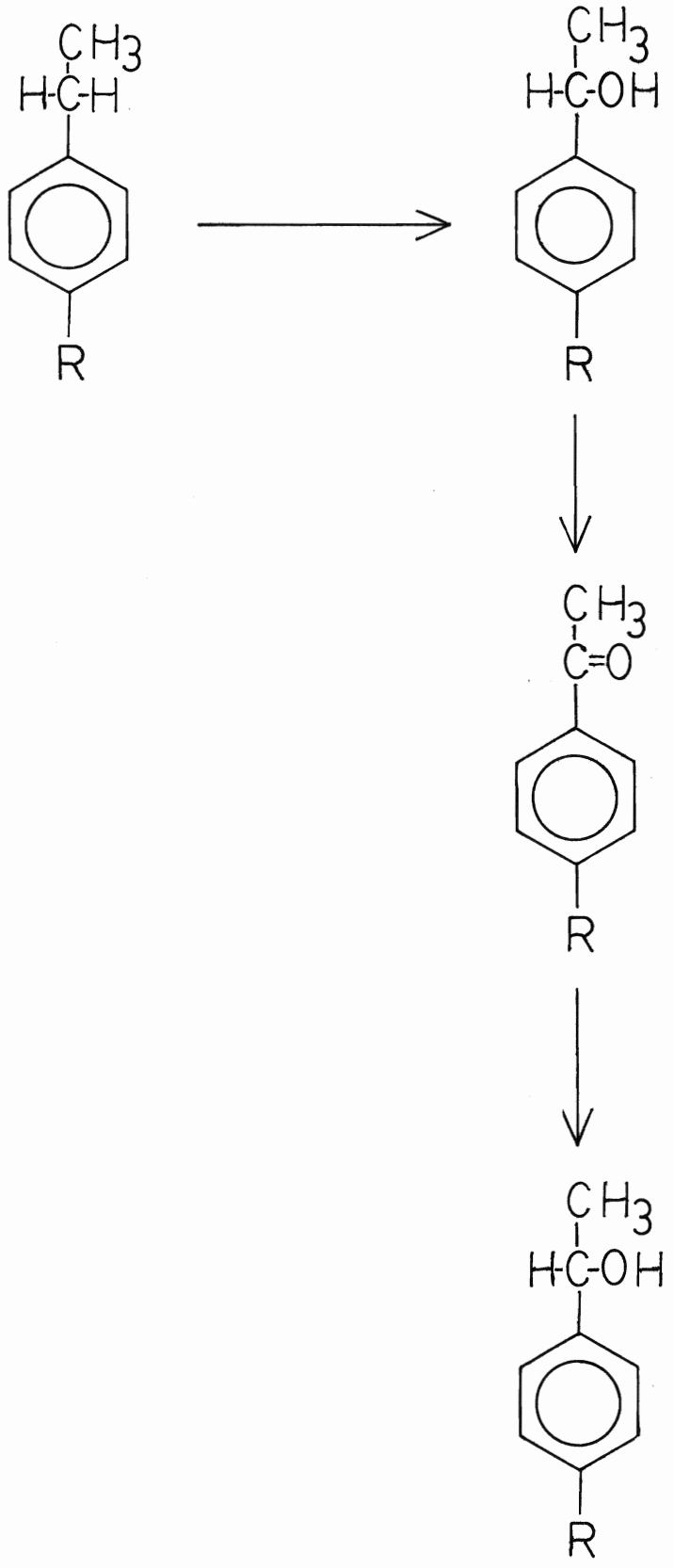
An alternate explanation for the inconsistent results obtained in this study may be as follows. That there may be two forms of cytochrome P-450, each catalysing the hydroxylation with opposite stereospecificity and the difference in rate of metabolism by the two forms gives rise to excess of one enantiomer. This metabolic pathway is independent of electronic and/or steric property of para substituent. To investigate this possibility the incubations with ethylbenzene were performed at different concentrations with the hope that it might change the rate of metabolism by two forms as each enzyme has its own specific K_m. The products so obtained have essentially the same optical purity. These results indicate that change of concentration of substrate in incubation medium does not alter the K_m for the microbial metabolism.

Unpublished results[194] from our laboratory show that the incubations of ethylbenzene at different temperatures indicate no change in enantiomeric excess of product, 1-phenylethanol. These two important observations do not support the idea of more than one form of cytochrome P-450 responsible for these hydroxylations to produce optically active alcohols. However, more experiments are required to support this observation. The possibility of different enzymes for different substrates, although remote, definitely needs certain investigations.

Alternatively, a quantitatively altered metabolic pathway could account for the discrepancies seen in the stereoselective hydroxylations of ethylbenzenes. It can be proposed that hydroxylation of these substrates occurs by three distinct steps (Fig. 39) and at least one or all three are stereoselective. The first step is the formation of 1-phenylethanol, the second step is the oxidation to produce acetophenone, and the third is the reduction to give optically active alcohol.

Some control experiments[195] conducted in our laboratory after the completion of this study revealed that incubation of p-diethylbenzene, 28c with M. isabellina for 24-48 hours gives 1-(p-ethylphenyl)ethanol, 51c , without any detectable amount of p-ethylacetophenone, 35c . When incubation was run for 72 hours, significant amount of p-ethylacetophenone, was obtained in addition to 1-(p-ethylphenyl)ethanol. In this connection it should be noted that it has been recently reported that transformation of fluoren-9-ol to

Figure 39. Proposed metabolic pathway for the hydroxylation of Alkylbenzenes



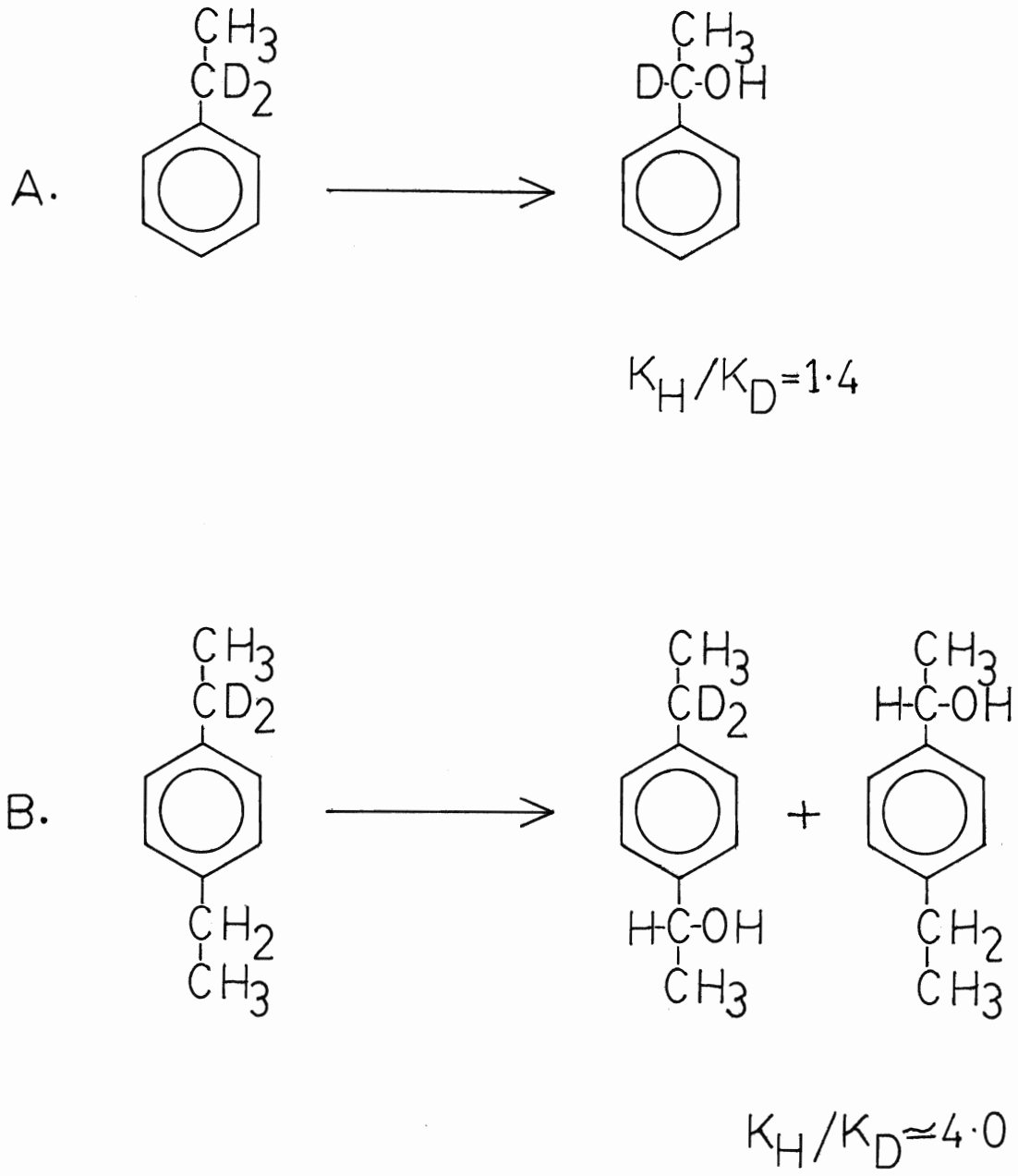
fluoren-9-one by liver microsomal preparations involves cytochrome P-450 oxygenase.[190] These observations support the view that acetophenone is formed from 1-phenylethanol and this reaction is most probably cytochrome P-450 dependent although not directly proved in the present case. The reduction of acetophenone to 1-phenylethanol proceeds with a very high degree of stereoselectivity has been shown in this study. It must, therefore, be assumed that this step, at least in part, contributes to the overall optical purity observed. In the absence of any information regarding the stereoselectivity of preceding steps, no definite conclusion can be drawn from this study.

Determination of optical purity of 1-phenylethanol obtained from 24 to 48 hours incubation of ethylbenzene and regular incubations of (R)-(+), (S)-(-) and (RS)-(±)-1-phenylethanol with M. isabellina would provide necessary information about the stereochemical approach of first two steps of the proposed metabolic pathway.

It may be relevant in this context that the hydroxylation of α,α-d_2-ethylbenzene aby M. isabellina provided 1-phenylethanol and the reaction was slower due to a deuterium isotope effect (k_H/k_D = 1.4). 1-Phenylethanol thus formed retained one deuterium atom (Fig. 40 A).[195] In a similar investigation, 1-(4-phenylethyl)-ethane-1,1-d_2 was used as a substrate and hydroxylation was observed to proceed with a significant deuterium isotope effect which was found to be: k_H/k_D = 4.0 (Fig. 40 B).[195]

From the above investigations, it is clear that fungal

Figure 40. Hydroxylation of α,α-d_2-ethylbenzene and 1-(4-phenylethyl)-ethane-1,1,d_2 by Mortierella isabellina



hydroxylation of ethylbenzene apparently proceeded by the loss of only one of the ∝-hydrogens of ethylbenzene. The KIE studies clearly support stepwise mechanism (Fig. 24 B). However, there still remained the question whether hydroxylation occurred by retention of absolute stereochemistry (front side displacement) or inversion of configuration (back side displacement). To answer this question, optically active (S)(+)∝-d-ethylbenzene has been prepared and metabolic investigations are currently in progress in our laboratory.[196]

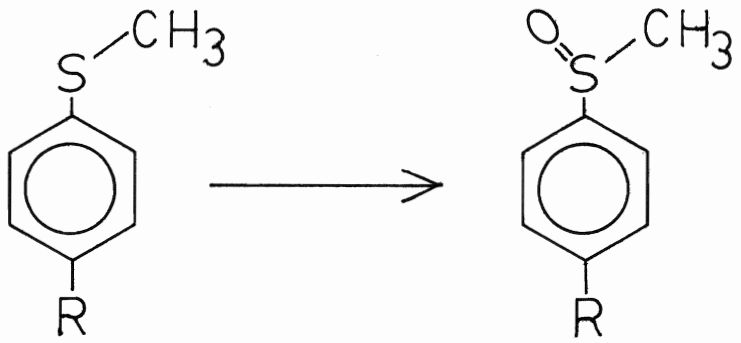
Formation of 2-arylethanol (in some cases) can be attributed to the same enzyme which is responsible for the production of 1-arylethanol. This assumption is based on the observation that the oxidation of para-substituted phenyl and benzyl methyl sulphides is catalysed by cytochrome P-450 present in M. isabellina [114] (Fig. 41, A and B).

The assignment of absolute configuration of the predominant enantiomers in Table 9 is based on the reported stereochemistry of chiral alcohols of known optical rotation in the case of 51a [88,89] and 51d .[98] Other absolute configurations are based on the reported observation that dextrorotatory methylaryl carbinols have R configuration.[176]

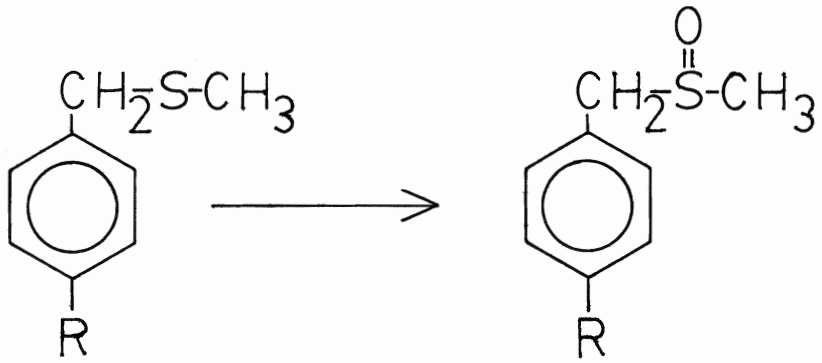
The observed optical rotations for 1-arylethanols were compared with the values reported in the literature. The values so obtained for enantiomeric enrichments sometimes differed from values obtained by using n.m.r. shift reagent method. The problem with these optical rotation measurements is that in some cases a trace amount of lipid

Figure 41. Sulphide oxidation by Mortierella isabellina

A.



B.



(from mycelia) was present in the sample even after repeated column chromatography and this lipid may have some optical rotation, thus affecting the accurate measurements. Thus, value obtained from the optical rotation measurements serve only as a rough check. However, the chiral shift reagent employed in the present study performed admirably in determining the enantiomeric excess of microbial products.

It is hoped that this study has demonstrated what an important and fascinating field is open in the research of fungal hydroxylations.

Future Work

Future prospect in this field is the research on these hydroxylations using *Helminthosporium sp.* This fungus is capable of the same sulphoxidation reactions as *M. isabellina*, but with opposite configuration and excellent stereoselectivity.[197] Therefore, it is possible that this fungus can provide better results.

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A P P E N D I X (I)

Dibenzothiophene, 1	Aldrich Chemical Co.
Dibenzopyrrole, 2	Chem. Service Media
Fluorene, 3	Aldrich Chemical Co.
9-Flurenone, 4	Aldrich Chemical Co.
Acridine, 5	Chem. Service
Phenanthridine, 6	Aldrich Chemical Co.
1:10-Phenanthroline, 7	May & Baker Ltd. (Eng.)
Benz[a]anthracene, 8	Prof. V. Snieckus
Benz[c]acridine, 9	Prof. V. Snieckus
Dihydronorharman, 10	Prof. D.B. MacLean
Triphenylphosphine, 12	Chem. Service
Dibenzo-1,4-dioxin, 13	Prof. M.S. Gibson's Lab.
5H-Dibenzo[a,d]cycloheptn-5-one, 14	Aldrich Chemical Co.
Dibenzosuberone, 15	Aldrich Chemical Co.
Thianaphthene, 16	Aldrich Chemical Co.
Alpha-Terthienyl, 17	Prof. P. Morand
Thiochroman-4-one, 18	Aldrich Chemical Co.
2-Phenylthiochroman-4-one, 19 [1]	Prof. I.W.J. Still
Thioxanthan-9-one, 20	Aldrich Chemical Co.
1-Methylthioxanthone, 21 [2]	Mr. I.D. Brindle
2-Methylthioxanthone, 22 [2]	Mr. I.D. Brindle
3-Methylthioxanthone, 23 [2]	Mr. I.D. Brindle
4-Methylthioxanthone, 24 [2]	Mr. I.D. Brindle

Canadine, 25	Prof. D.B. MacLean
Tetrahydropalmatine, 26	Prof. D.B. MacLean
Corydaline, 27	Prof. D.B. MacLean
Ethylbenzene, 28a	Eastman Organic Chemicals
p-Ethyltoluene, 28b	Aldrich Chemical Co.
p-Diethylbenzene, 28c	Aldrich Chemical Co.
p-Methoxyethylbenzene, 28d	Dr. H.L. Holland
p-Fluoroethylbenzene, 28e	Columbia Organic Chemicals Co.
p-Chloroethylbenzene, 28f	ICN Pharmaceuticals, Inc.
p-Ethylaniline, 28h	Aldrich Chemical Co.
p-Ethylphenol, 28j	Aldrich Chemical Co.
n-Propylbenzene, 29	Eastman Organic Chemicals
Acetophenone, 33a	BDH Laboratory regents
p-Methylacetophenone, 33b	Aldrich Chemical Co.
p-Ethylacetophenone, 33c	Aldrich Chemical Co.
α,α,α-Trifluoacetophenone, 36	Aldrich Chemical Co.

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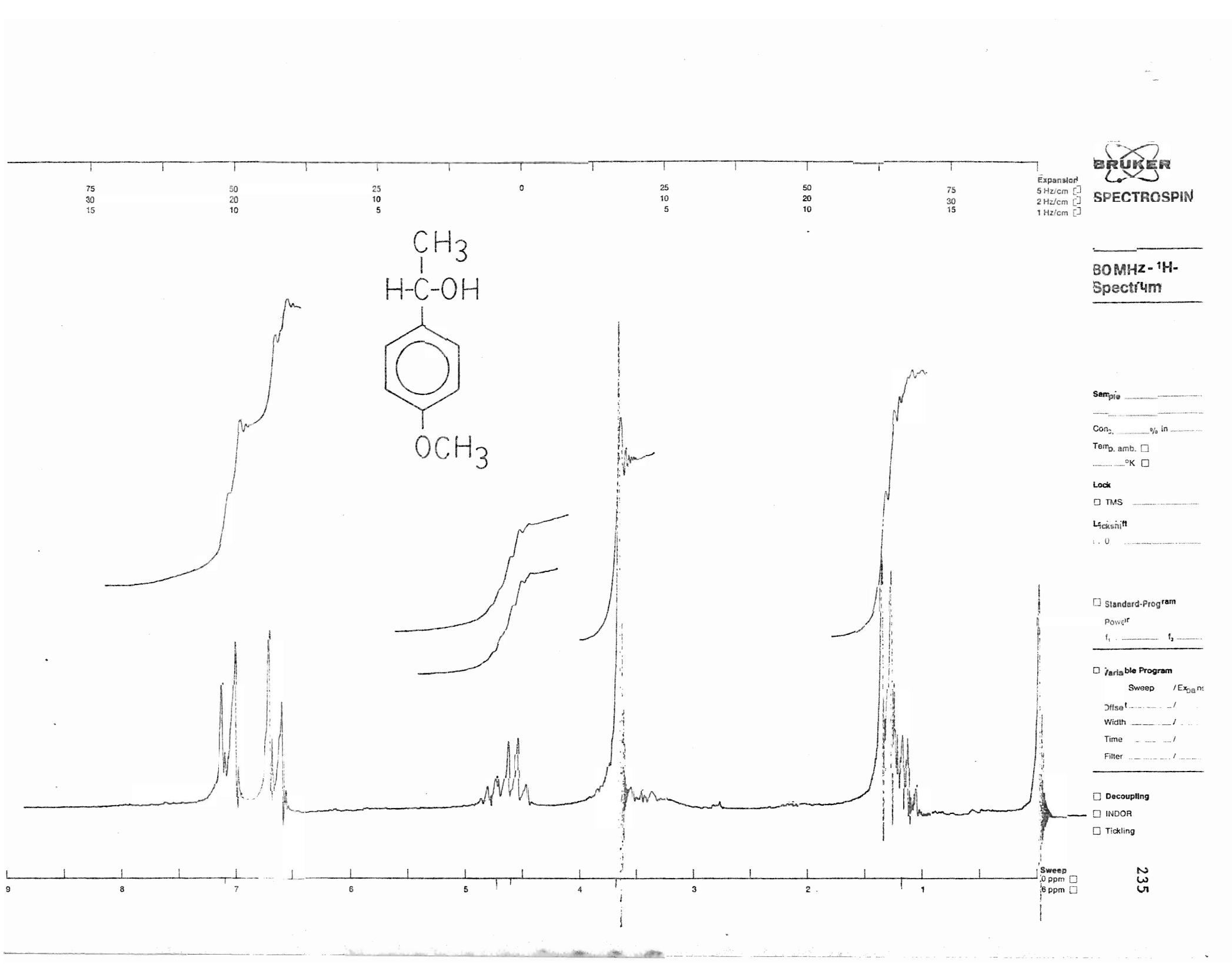
A P P E N D I X (II)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Sample
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Variable Program
Sweep
Offset
Width
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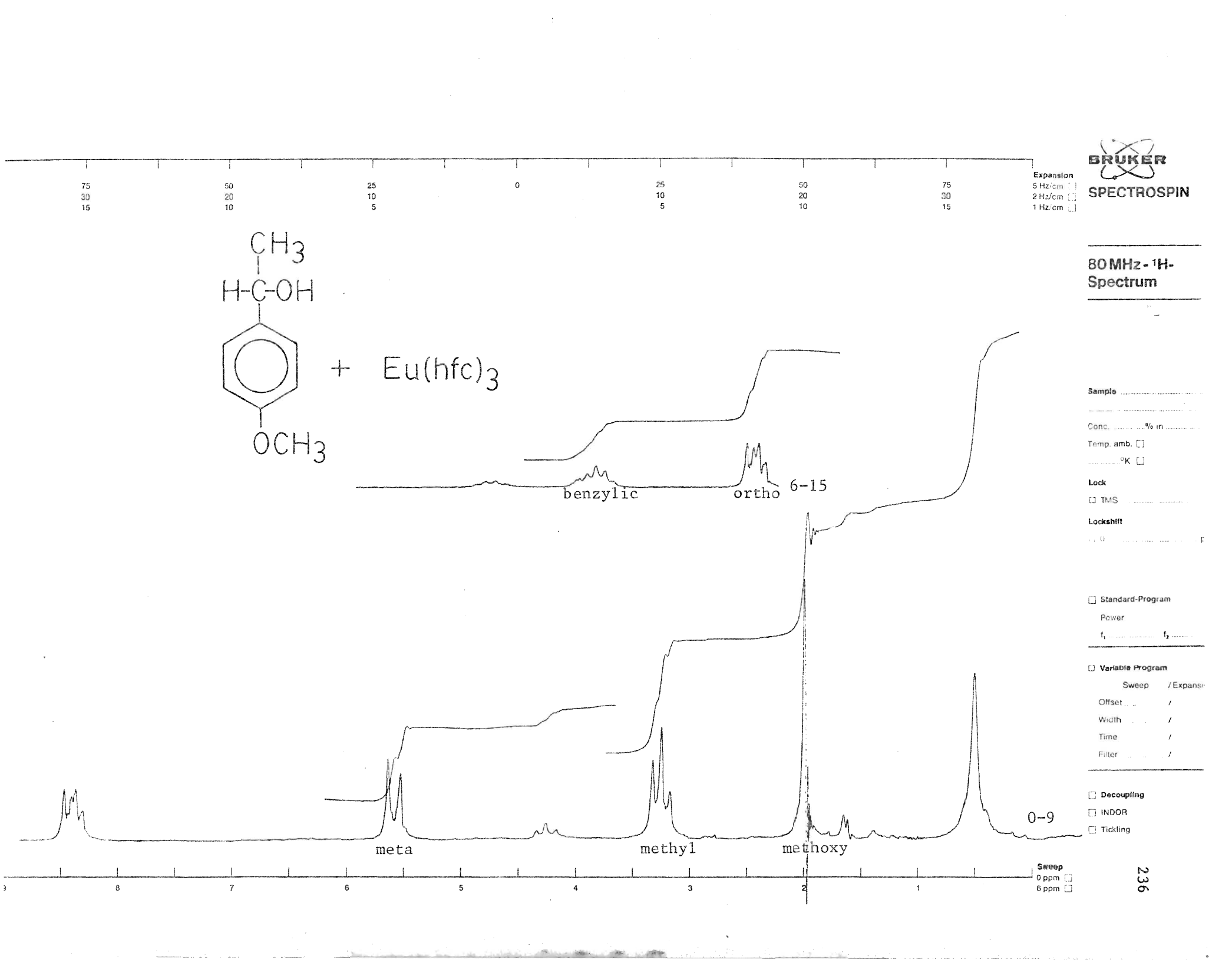
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80 MHz - 1H-Spectrum
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+ Eu(hfc)3
benzylic
ortho
6-15
meta
methyl
methoxy
0-9
Sample
Conc. % in
Temp. amb.
°K
Lock
TMS
Lockshift
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Filter
Decoupling
INDOR
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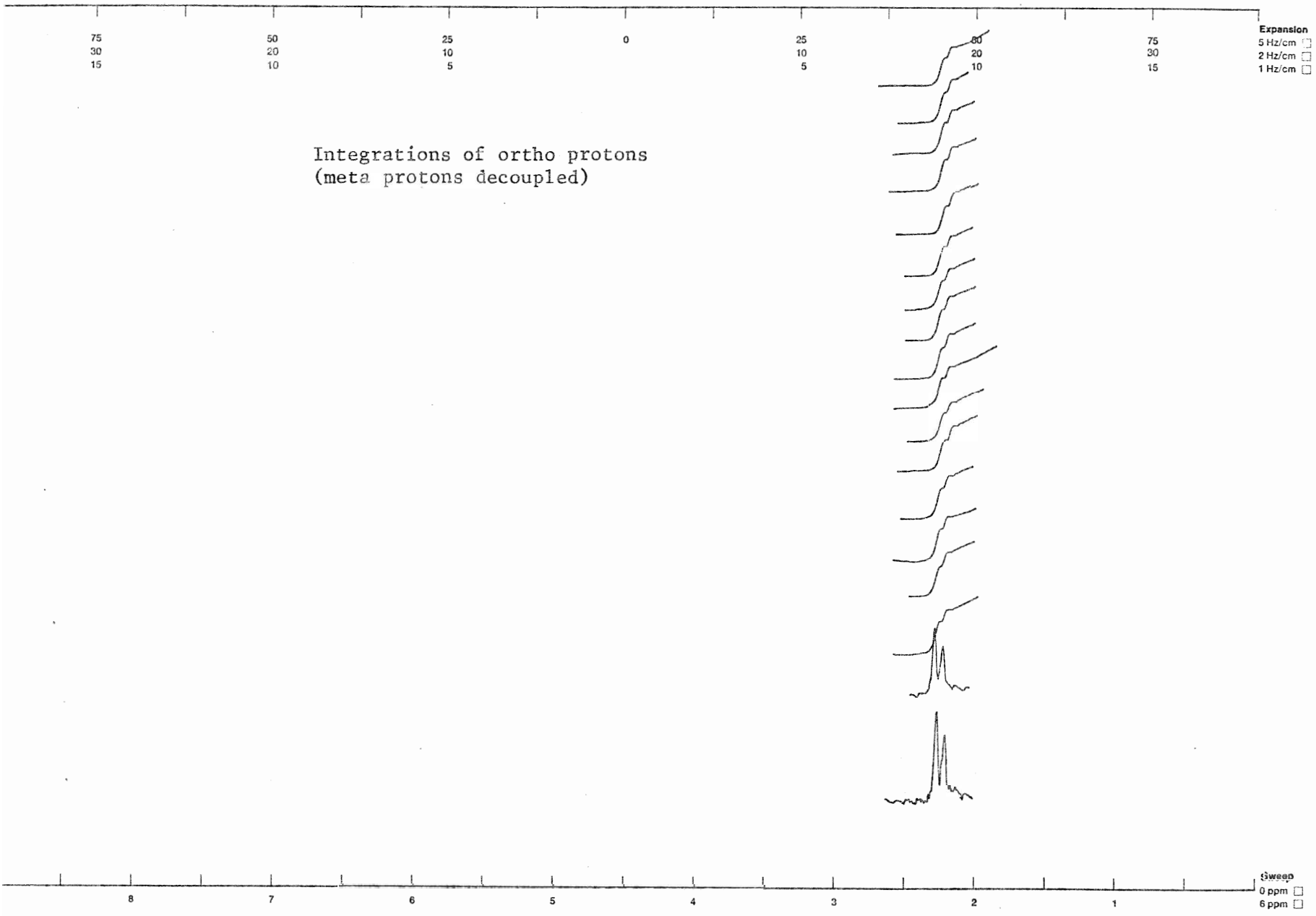
236



Integrations of ortho protons
(meta protons decoupled)

BRUKER
SPECTROSPIN

80 MHz - ^{1}H-
Spectrum

Sample .....

Conc. .....% in .....

Temp. amb. ☐

.....°K ☐

Lock

☐ TMS .....

Lockshift

. . 0 ..... ppm

☐ Standard-Program

Power

f_1 ..... f_2 .....

☐ Variable Program

Sweep / Expansion

Offset ..... /

Width ..... /

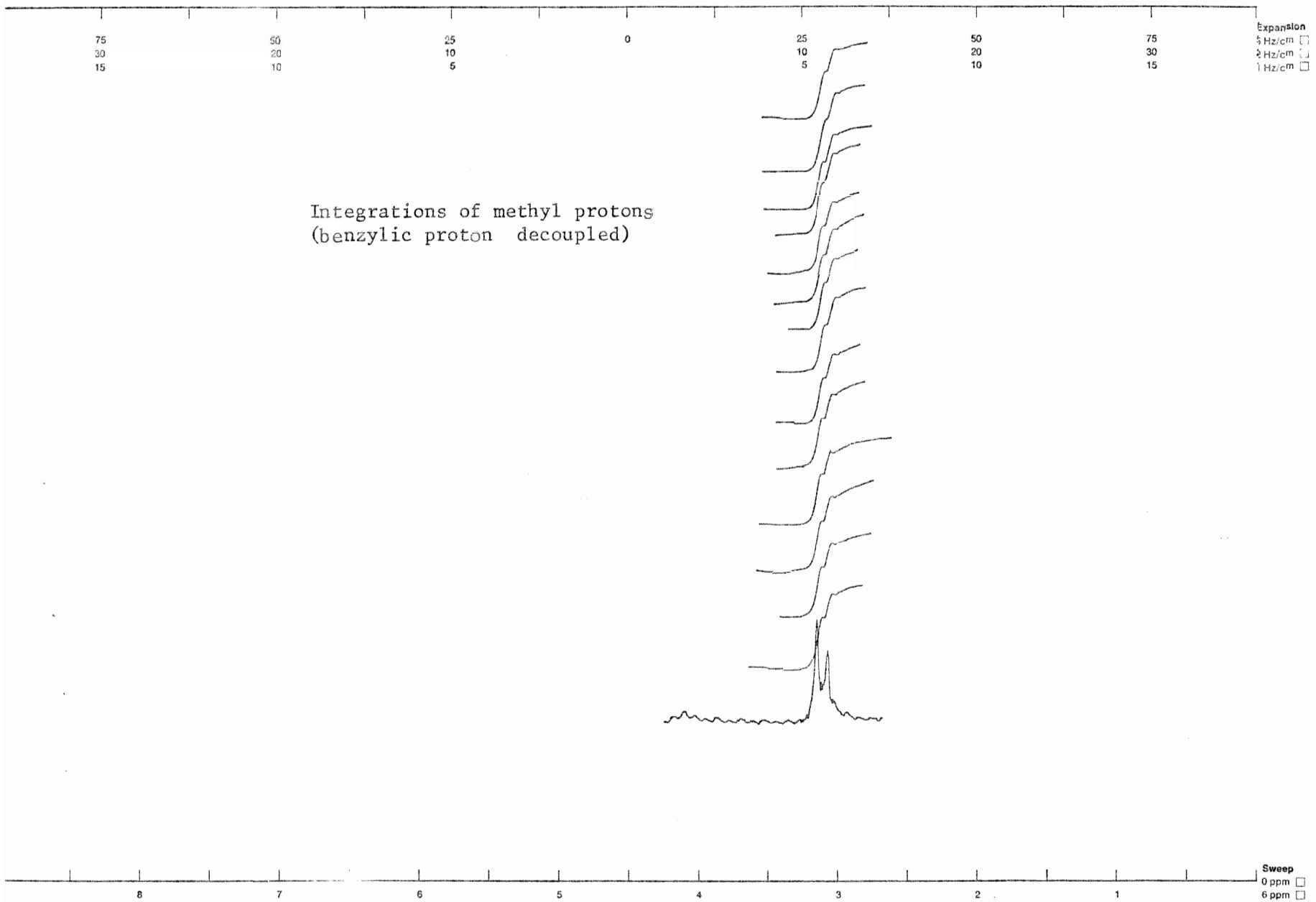
Time ..... /

Filter ..... /

☐ Decoupling

☐ INDOR

☐ Ticking



BRUKER SPECTROSPIN

80 MHz - ^{1}H-Spectrum

Sample .....

Conc. ..... % in .....

Temp. amb. ☐

..... °K ☐

Lock

☐ TMS .....

Lockshift

... 0 ..... F

☐ Standard-Program

Power

f_1 ..... f_2 .....

☐ Variable Program

Sweep / Expansi

Offset ... / ...

Width ... / ...

Time ... / ...

Filter ... / ...

☐ Decoupling

☐ fNDOR

☐ Tickling