A S P E C T S O F T H E
E N V I R O N M E N T A L C H E M I S T R Y
O F M E T H O X Y C H L O R

BY

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A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
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ABSTRACT

Preliminary investigations on the Environmental Chemistry of Methoxychlor [2,2-bis (*p* - methoxyphenyl) -1,1,1- trichloroethane], a potential DDT replacement, have been conducted with emphasis on its degradation by micro-organisms.

Literature reports on the microbial degradation of methoxychlor are scarce, and in those reports the major emphasis is on the interaction of this pesticide with bacteria. Therefore, studies concerning the interaction of micro-organisms with methoxychlor were initiated. The micro-organisms used were the common soil fungi *Trichoderma viride*, *Mortierella isabellina*, *M.pusilla*, and the aquatic fungus *Saprolegnia parasitica*. Also because of anomalous results reported in the literature on the possible biodegradation of methoxychlor by a bacterium, *Klebsiella pneumoniae*, this aspect of the environmental fate of methoxychlor was reinvestigated.

Known metabolites of methoxychlor required for this study were synthesized. Some of the synthetic methods were slightly modified so as to improve yields or facilitate purification of the products. Also to facilitate the identification of possible degradation products, the spectroscopic properties (MS and NMR) of

these various methoxychlor derivatives were studied. A gas chromatographic method was developed for the analyses of these compounds and included the silylation of the phenolic compounds.

Little is known about the fungitoxicity of methoxychlor or its metabolites, thus such fungitoxicity studies were carried out. It was found that the dihydroxy methoxychlor derivatives were more toxic than the dimethoxy methoxychlor derivatives, towards the fungi used. In fungal metabolism studies with methoxychlor, *M.isabellina*, *M.pusilla*, and *S.parasitica* were found to be capable of partially degrading methoxychlor, by effecting the ether cleavage of the methoxy groups in the molecule. *T.viride* did not effect a similar alteration of the methoxychlor molecule but appears to produce at least one metabolite of methoxychlor. It is suggested that this metabolite is a glycoside. No evidence was found to indicate that the fungi studied, attacked the central part of the methoxychlor molecule.

K.pneumoniae was found to be capable of the reductive dechlorination of methoxychlor and also its hydroxy derivative [2,2-bis (*p* - hydroxyphenyl)-1,1,1- trichloroethane]. It was also established that this bacterium does not effect the cleavage of the methoxy groups in methoxychlor.

While the bacterial system, i.e. *K.pneumoniae*, allowed quantitative analysis of the various extracts, the fungal mycelium of the species used strongly adsorbed the various methoxychlor derivatives, which interfered with the quantitative analysis in these cases.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

The potential environmental hazards caused by the extensive use of toxic and persistent organochlorine insecticides, have been a major concern to Environmental Scientists for some time. One of these compounds is the very persistent 2,2-bis (*p* - chlorophenyl)-1,1,1-trichloroethane, (DDT, structure I, p. 4). Its use as an insecticide has benefited mankind tremendously and must not be underestimated, as it was used successfully in controlling insect vectors of many human diseases (e.g. malaria, typhus, and plague). However, DDT and its major metabolites, 2,2-bis (*p* - chlorophenyl) -1,1- dichloroethylene, (DDE,II) and 2,2-bis (*p* - chlorophenyl)-1,1- dichloroethane, (DDD,III), because of their persistence, have been found to accumulate in the biosphere (1).*

Studies with DDT, DDE and DDD have shown these compounds to be efficient inducers of microsomal oxidase enzymes in the vertebrate liver. Nevertheless, it appears that these compounds are environmentally injurious because of the stability of the aryl chlorine bonds,

* Arabic numerals in parentheses indicate literature references on p.128.

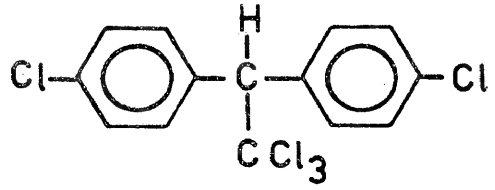
which are not readily attacked by such enzymes. These compounds are stored in tissue lipids and are not readily metabolised or excreted. Thus, there is a need for the replacement of DDT by compounds which are effective as insecticides and are also biodegradable. Moreover, if such replacements interact with higher organisms, they should be rapidly biodegraded and excreted, and the resulting metabolic products should not be persistent in the environment as is the case with DDT and its major metabolites (DDE,II and DDD,III).

Prill *et. al.* (2) prepared a number of DDT analogues including some alkoxy derivatives, and tested the effectiveness of these against mosquito larvae and houseflies. These authors found a few of the alkoxy analogues to be similar to DDT with respect to their ability either to knockdown or kill the insects. Thus, their results indicated that some of the alkoxy analogues could be useful in insect control.

2,2-Bis (*p* - methoxyphenyl)-1,1,1- trichloroethane, (Methoxychlor,IV) was one of the alkoxy DDT analogues prepared by Prill *et. al.* (2) which has become a candidate for the replacement of DDT as a biodegradable insecticide (1). This analogue contains methoxy groups which are sites for attack by multifunction oxidase enzymes. There are no such sites in the DDT molecule and methoxychlor,(IV) has already been used to replace DDT

in several instances, including the control of the elm bark beetle vector associated with Dutch Elm disease (1) and in the control of many biting insects (3). Other compounds mentioned as potential replacements for DDT include 2,2-bis (*p*- methylthiophenyl)-1,1,1- trichloroethane, (Methiochlor,V) and 2,2-bis (*p*- methylphenyl)-1,1,1- trichloroethane, (methylchlor,VI) (4).

Since Kapoor *et. al.* (1) suggested that methoxychlor,(IV) could potentially replace DDT, it has received much attention. Methoxychlor was first synthesized in 1893 (5) and its insecticidal properties were described by Lauger *et. al.* (6), together with those of DDT. Although its insecticidal action is not as strong as that of DDT, its application for effective insect control has increased since the use of DDT was restricted in 1969 (7). In Canada many mixtures containing methoxychlor are registered for various usages and this pesticide was used on an experimental basis for the control of biting flies, particularly in the northern regions where it was used as a black fly larvicide (8). However, the use of methoxychlor has not yet been extensive, partly because of its higher cost, and partly because its environmental impact has not been fully evaluated. The literature on methoxychlor has been reviewed by Gardner and Bailey (7), and these authors stated that



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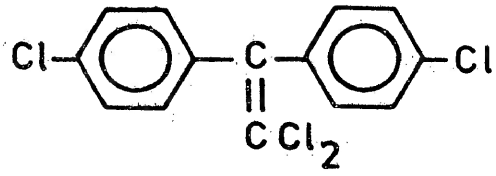

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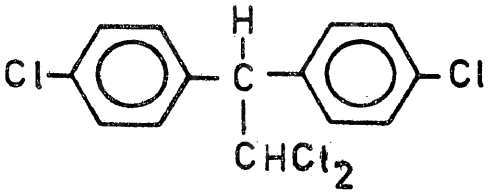

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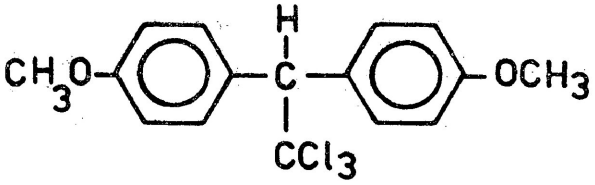

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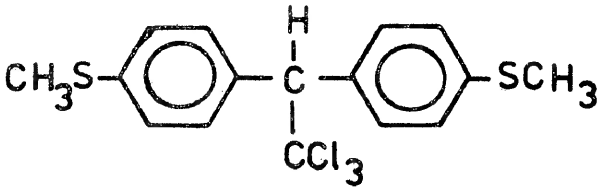

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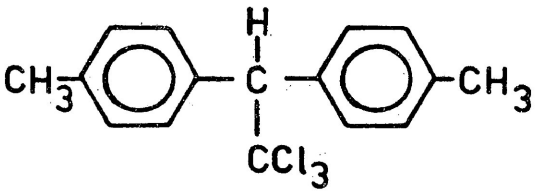

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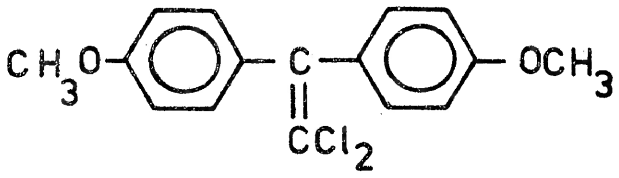

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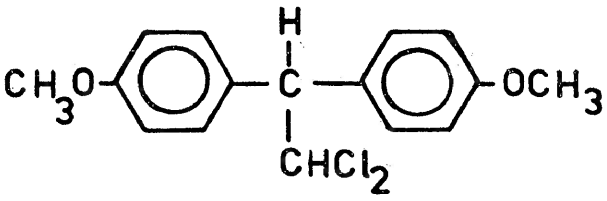


VIII

present knowledge concerning methoxychlor's impact upon the environment is in an early stage. Kapoor *et. al.* (1) also stated that, despite twenty-five years of use, little specific information concerning metabolic pathways, elimination from organisms and the fate of this pesticide in the ecosystem, is known. Consequently an assessment of the environmental impact of this pesticide cannot be made until such studies are further advanced. Major areas where further study is required include movement in the environment, persistence, bioaccumulation, biodegradation, and economic value.

Therefore, the objective of the studies described in this thesis is to improve the present state of knowledge concerning methoxychlor, with major emphasis on its biodegradability by micro-organisms.

A comparison of methoxychlor with DDT, suggests that the former is only moderately persistent in the environment and is less toxic to higher life forms. This is illustrated by the fact that the LD_{50} for male rats fed orally with DDT is 200mg./kg. body weight while for methoxychlor this value is 6000mg./kg. body weight (9). Furthermore, mammals and birds presumably are able to metabolise methoxychlor more readily than DDT, because the multifunction oxidase enzymes capable of degrading methoxychlor are readily available in these organisms (7). For example, Weikel (10) injected

rats with ^{14}C labelled methoxychlor intravenously and found the label in polar, water-soluble metabolites. Methoxychlor itself was not excreted.

Another aspect of methoxychlor which requires further study follows from the statement that methoxychlor is strongly absorbed on solids suspended in water, resulting in a potential for high residue concentrations (8). This could adversely affect organisms in close contact with such residues (e.g. micro-organisms). Fredeen *et. al.* (8) also state that these concentrations are washed away with time, but quantitative information to confirm such a statement, was not provided by these authors.

Thus, if methoxychlor is to be used extensively as a substitute for DDT in pest control in the near future, it becomes important to demonstrate that biologically significant quantities of this compound, or products of its metabolism, do not accumulate in terrestrial or aquatic ecosystems. The removal of such substances from the biosphere must be assigned to two processes, photodecomposition and biodegradation. MacNeil *et. al.* (11) investigated the photolysis of methoxychlor. These authors determined quantitatively, the two major photodecomposition products, 2,2-bis (*p*- methoxyphenyl)-1,1- dichloroethylene, (methoxychlor olefin,VII) and 2,2-bis (*p* - methoxyphenyl)-1,1-dichloroethane (methoxydichloromethyl derivative, VIII),

(see p.4), as their π-complexes with the electron-acceptor reagent 2,4,7-trinitro-9-fluorenone *in situ* on Silicagel thin layer chromatograms by UV-VIS reflectance spectroscopy. Zepp *et. al.* (12) have carried out an investigation of the photodecomposition of methoxychlor in aquatic systems and found that the major photodecomposition product in distilled water was the methoxychlor olefin,(VII). Biodegradative studies concerning methoxychlor metabolism by rats have been reported (1,10). Other studies with mammals and methoxychlor are concerned almost exclusively with toxicity, and little mention has been made about biodegradation (7). There is also very little in the literature on the subject of degradability by micro-organisms.

Bioaccumulation of methoxychlor by various organisms is another area that requires further study. In most of the organisms studied, methoxychlor has been found to be less bioconcentrated than DDT with some exceptions (7). There are also several examples where, in higher terrestrial organisms, bioaccumulation is not normally observed, probably due to fairly rapid metabolism and excretion of metabolites (7). Accumulations of this pesticide in fish with concentration factors ranging from 40 to 1500, reflect the slower metabolism of methoxychlor compared to that in mammals (7). The

concentration factors for DDT range up to 84,000. Several authors (13, 14) have reported that molluscs tend to accumulate methoxychlor. Reinbold *et. al.* (13) have also reported that a water snail (*Physa* species) has a concentration factor of 2600 for methoxychlor as compared to 1200 for DDT, but this high methoxychlor concentration does not appear to be lethal to this organism. There is evidence of extensive accumulation of methoxychlor by phytoplankton, but quantitative data is not available (7). Reinbold *et. al.* (13) have also reported that the aquatic organism *Daphnia magna* could be responsible for some of the pesticide biomagnification, having a concentration pattern similar to that displayed by fish. In the terrestrial ecosystem, high levels of methoxychlor have been found in three species of earthworm (15), however the extent of bioconcentration is not known. Thus, organisms which feed exclusively on those organisms that can accumulate methoxychlor, could be subjected to lethal doses of this pesticide. Johnson and Kennedy (16) have reported that *Aerobacter aerogenes* and *Bacillus subtilis* accumulated methoxychlor when incubated with a concentration of 1 g./l. of labelled material. They suggested that this accumulation was merely a passive process since accumulation was also observed with autoclaved cells. The bioaccumulation phenomenon thus

represents an important aspect in the fate of this pesticide. It should be noted that many of these bioaccumulation studies were carried out using radiolabelled (^{14}C or ^{3}H) methoxychlor. However, such measurements do not always explain whether it is methoxychlor itself that is accumulated, or whether the pesticide first undergoes chemical modification followed by the accumulation of labelled metabolites. Thus, it becomes clear from the foregoing that a major aspect of methoxychlor chemistry requiring further study is its biodegradation, particularly by micro-organisms.

For the purposes of this thesis, the interaction of methoxychlor and some of its known metabolites with some common soil and aquatic fungi and a bacterium were chosen for further study.

Metabolic studies with methoxychlor and micro-organisms are limited to a few bacteria and algae. Castro and Yoshida (17) investigated the degradation of methoxychlor in Philippine soil samples at 30° under upland (80% moisture) and flooded (submerged) conditions. Methoxychlor disappeared in both cases, but these authors did not attempt to identify any metabolites or organisms. Sethunathan and Yoshida (18) showed that 10ppm labelled methoxychlor was degraded after two hours of incubation with a *Clostridium* species. They did not

isolate or identify any degradation products. Mendel *et. al.* (19) reported that the bacterium *Aerobacter aerogenes*, after longterm incubation with this pesticide (114hr.) degraded methoxychlor to its olefin,(VII) in two of nine experiments and to the methoxydichloromethyl derivative,(VIII) (see p.4) in only one of these experiments. In the remaining fermentations, with one exception, these authors also stated that in general, a considerable amount of the pesticide is lost during incubation. A possible explanation for such a statement is the fact that they used benzene for the extraction of their cultures, while present studies have shown (see p.35) that several of the phenolic methoxychlor derivatives are virtually insoluble in benzene.

A study on the loss of five pesticides, including methoxychlor, from cultures of several planktonic algae was reported by Butler *et. al.* (20a). They found that the amount of methoxychlor recovered ranged from (79%) to (20%) in the cultures of twenty-one different species. No attempt was made to identify possible metabolites, and these authors could not conclusively state that metabolism was responsible either totally or in part for the pesticide losses. The same authors also did a growth study using methoxychlor and a number of planktonic algae (20b). They used concentrations of 0.001 and 0.01ppm of methoxychlor and found that growth

of the algae in the presence of methoxychlor was not significantly different from that in the controls. Other studies with micro-organisms deal specifically with toxicity in terms of inhibition of growth (21, 22, 23, 24) and in these studies no mention is made of metabolism of methoxychlor by micro-organisms. In fact, in one of these reports Poorman (22) treated cultures of *Euglena gracilis* (Klebs) with 10, 50 and 100ppm methoxychlor for one week and growth was stimulated. However, it must be observed that planktonic algae in the natural environment would not normally be exposed to such high concentrations of methoxychlor.

In view of the close chemical relationship between methoxychlor and DDT, and since considerable more work has been done with DDT, a brief discussion of the environmental chemistry of DDT is relevant as a background for the present study.

DDT is the best known, the cheapest and the most effective of the synthetic organochlorine insecticides. The properties that have made it successful as an insecticide are chemical stability, low vapour pressure (1.5×10^{-7}mm. at 20°), low water solubility (2ppb) and high lipid solubility (approx. 100,000ppm) (4). This insecticide has been extensively used since 1943, and it has caused increasing concern regarding its longterm bioactivity and environmental persistence. Ironically

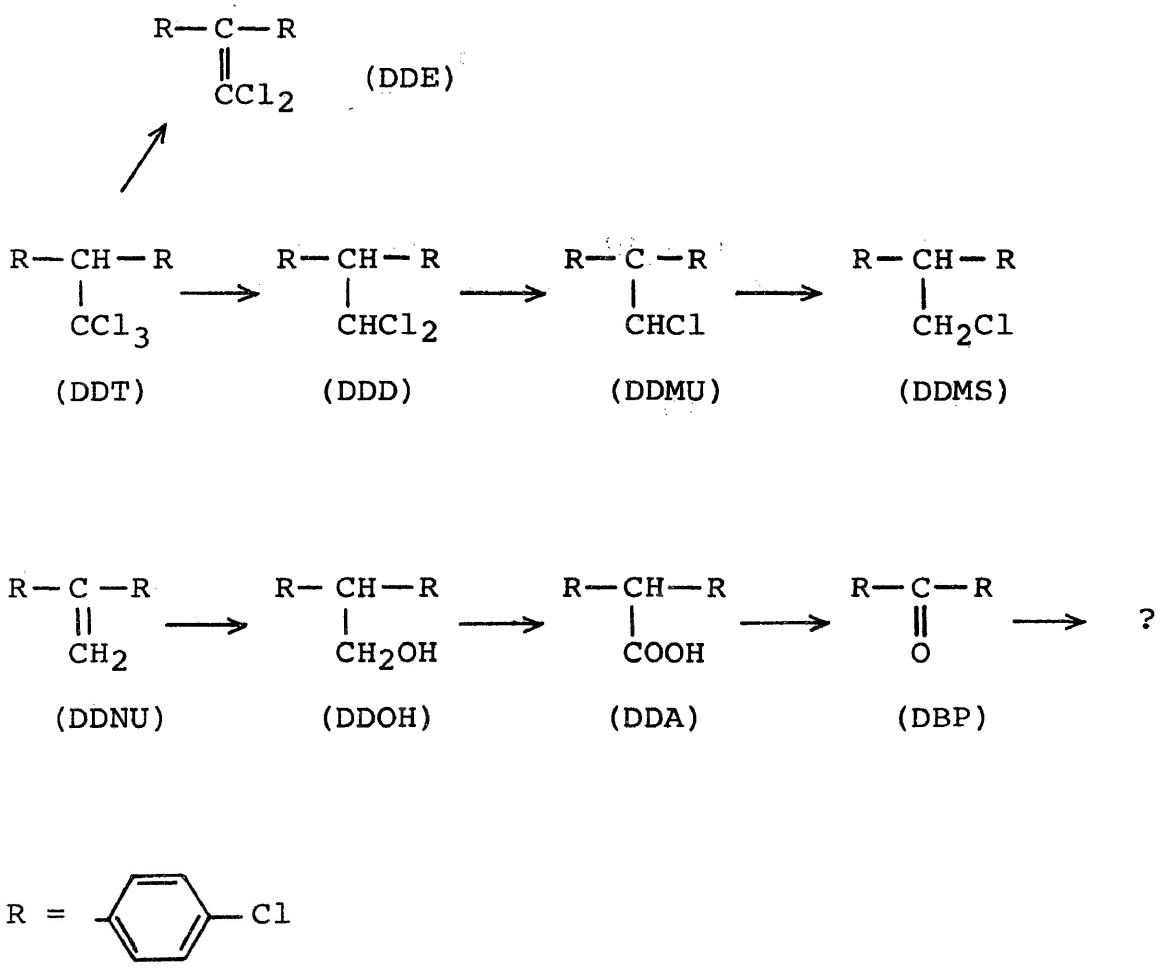
the same properties that are responsible for its success as an insecticide, have also made it the classic example of an environmental pollutant. DDT is found almost everywhere in a variety of organisms in both inland and marine waters at concentrations from ppt to ppb levels (25). Both by direct absorption and biological magnification it may be concentrated to levels much higher than that found in natural waters. It has been found in oysters up to 5.4ppm, whales to 6ppm, tuna to 2ppm and petrels to 10ppm (9). High levels in raptorial birds have deleterious effects on their reproductive capacity. Such levels have been suggested as the cause of abnormal calcium metabolism affecting egg production (26). The environmental problems caused by DDT are further compounded by the formation of its primary metabolites, i.e. DDE,(II) produced by the attack of the enzyme DDT-ase on the α-carbon of DDT (4) and DDD, (III) formed by reductive dechlorination (27). These DDT derivatives have physical properties similar to DDT and as a result are as ubiquitous as the parent compound. However, the concentrations of DDT, DDE and DDD which are found in the environment vary and DDE, which is not insecticidal is usually present in the highest concentration as it is environmentally more stable than DDT. DDD is susceptible to further degradation and is therefore found in low concentrations in the

environment.

Peterson and Robison (28) postulated a possible metabolic pathway for DDT metabolism when they recovered several metabolites from rats fed DDT orally. This pathway has been confirmed by the studies of Wedemeyer (29) who investigated the dechlorination of DDT by *Aerobacter aerogenes*, and identified seven metabolites, including DDD. The proposed sequence is presented in Scheme 1. Juengst and Alexander (30) investigated the conversion of DDT to water soluble products by micro-organisms, to further unravel its metabolic pathway. They did not achieve identification of these metabolites which were probably formed by further degradation of DDD. These authors used bacteria isolated from sea water and marine sediments.

Kapoor *et. al.* (1) has established that methoxychlor is susceptible to biodegradative reactions at two sites within the molecule. First, a series of reactions, analogous to those for DDT (see Scheme 1) may lead to the olefin and the dichloromethyl derivative. In addition to the reactions possible for DDT, the cleavage of the aryl methoxy groups may lead to similar series of methoxy/hydroxy and/or dihydroxy compounds. It follows that a large number of compounds may possibly result from methoxychlor metabolism which is presented in Scheme 2. To date, not all compounds in Scheme 2

Scheme 1. Suggested metabolic pathway for the biodegradation of DDT (see p.15 for legend).



Scheme 1, Legend

DDT: 2,2-bis (*p*- chlorophenyl)-1,1,1- trichloroethane.

DDE: 2,2-bis (*p*- chlorophenyl)-1,1- dichloroethylene.

DDD: 2,2-bis (*p*- chlorophenyl)-1,1- dichloroethane.

DDMU: 2,2-bis (*p*- chlorophenyl)-1- monochloroethylene.

DDMS: 2,2-bis (*p*- chlorophenyl)-1- chloroethane.

DDNU: unsym-bis (*p* - chlorophenyl) ethylene.

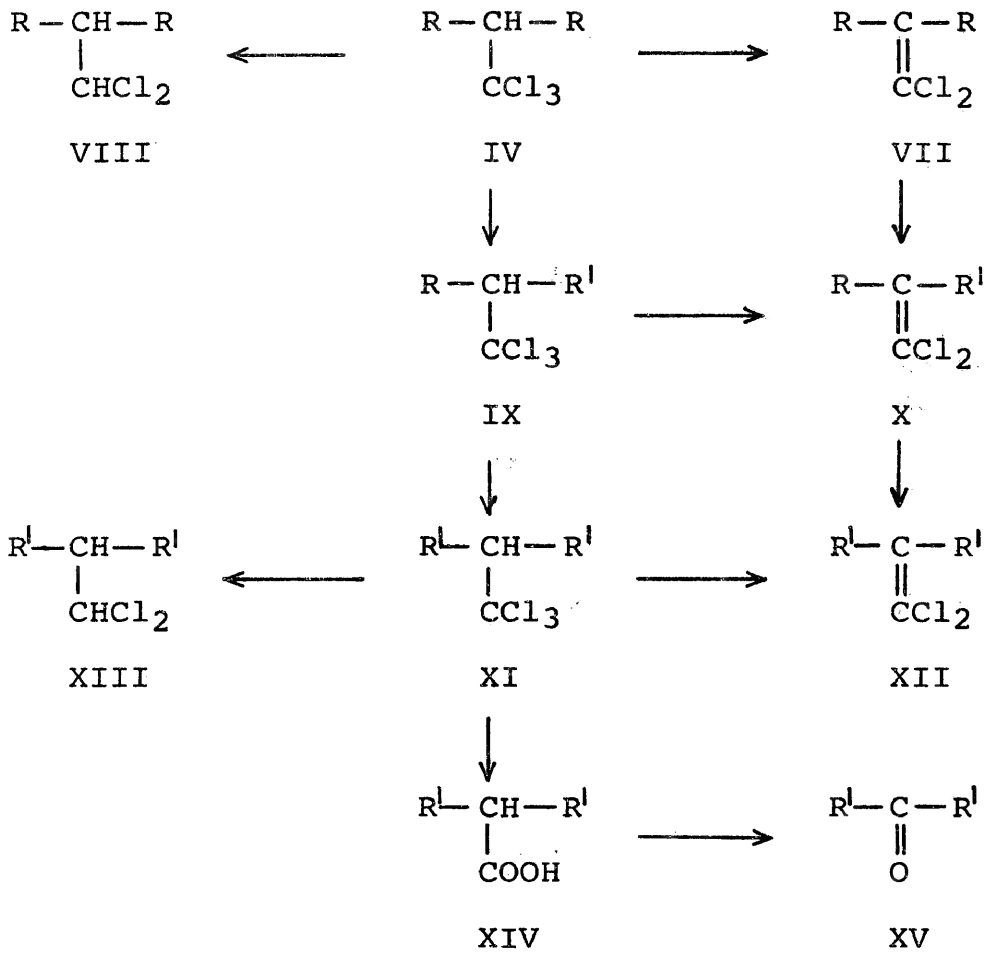
DDOH: 2,2-bis (*p*- chlorophenyl) ethanol.

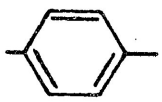
DDA: 2,2-bis (*p*- chlorophenyl) acetic acid.

DBP: 4,4' - dichlorobenzophenone.

Scheme 2. Possible metabolic reactions of methoxychlor.

(see p. 17 for legend)



R =  OCH_3 , R' =  OH

Scheme 2, Legend

IV. 2,2-bis (*p* - methoxyphenyl) 1,1,1- trichloroethane.

VII. 2,2-bis (*p* - methoxyphenyl) 1,1- dichloroethylene.

VIII. 2,2-bis (*p* - methoxyphenyl) 1,1- dichloroethane.

IX. 2- *p* - methoxyphenyl-2- *p* - hydroxyphenyl-1,1,1- trichloroethane.

X. 2- *p* - methoxyphenyl-2- *p* - hydroxyphenyl-1,1- dichloroethylene.

XI. 2,2-bis (*p* - hydroxyphenyl)-1,1,1- trichloroethane.

XII. 2,2-bis (*p* - hydroxyphenyl)-1,1- dichloroethylene.

XIII. 2,2-bis (*p* - hydroxyphenyl)-1,1- dichloroethane.

XIV. 2,2-bis (*p* - hydroxyphenyl) acetic acid.

XV. 4,4' - dihydroxybenzophenone.

have been isolated as metabolies from higher organisms and some also result from photochemical alteration of the methoxychlor molecule (11,12). It follows that a comprehensive study of the environmental impact of methoxychlor requires the availability of these known or anticipated metabolites. Consequently the present thesis includes a discussion of the synthetic chemistry of methoxychlor and its derivatives used throughout this study and also a report of their preparations according to published methods, modifications being used where appropriate.

Although these syntheses are discussed in detail in Chapter 2 (see p.23), one aspect of the chemistry of hydroxychlor, (XI,Scheme 2, p.16) must be mentioned here and concerns the solubility of this compound in non-polar solvents such as benzene. This has significance with respect to the work done by Mendel *et. al.* (19) with the bacterium *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) as described previously (see p.10). A reinvestigation of this interaction of methoxychlor and *K.pneumoniae* is a part of this thesis which has now resulted in the elucidation of a metabolic pathway for methoxychlor degradation by this bacterium (see Chapter 3).

From present literature, it appears that residue analysis is exclusively concerned with methoxychlor itself. McCully and McKinley (31) determined quantitatively,

a number of chlorinated pesticide residues in fat, using electron capture gas chromatography. Methoxychlor was one of the residues and the column stationary phase was a mixture of silicones (4% SE-30 methyl silicone + 6% QF-1 fluorosilicone).

Chau and Lanouette (32) developed a method using a derivative formation by the solid matrix, alumina/KOH and alumina/potassium *tert*-butoxide, for the confirmation of several chlorinated pesticides by gas chromatography, including methoxychlor. This method could be used to determine methoxychlor quantitatively.

Other methods used for analysing methoxychlor involved ^{14}C labelled compounds (1) and the use of the complexing electron - acceptor reagent 2,4,7-trinitro-9-fluorenone (TNF) (11) which was also used to determine methoxychlor olefin,(VII) and the methoxydichloromethyl derivative,(VII) (see p. 4). In all these reports, no mention is made of the phenolic derivatives of methoxychlor. Kapoor *et. al.* (1) are the only workers who have included some phenolic derivatives in their investigations and they developed a thin layer chromatography (TLC) technique for the qualitative identification of methoxychlor and some of its metabolites including a number of the phenolic derivatives. This technique has been utilized for preliminary identification of both phenolic and non-phenolic methoxychlor derivatives

in certain sections of this thesis.

From the preceding it was therefore necessary to develop a quantitative method for the determination of the phenolic methoxychlor metabolites. This was achieved using gas liquid chromatography (GLC) and the derivatization of the phenolic derivatives. The development of the technique is part of the present investigation.

There is virtually no information in the literature concerning biodegradation of methoxychlor by fungi. The only report found so far is a toxicity study by Richardson and Miller (21) who reported that methoxychlor is weakly fungitoxic to *Rhizoctonia solani* (Kühn) inhibiting growth by twenty percent. They did not however indicate whether this fungus could metabolise this compound. Since fungi, both terrestrial and aquatic species, could make an important contribution to the biodegradation of methoxychlor, it was decided that these organisms must be studied in more detail. Fungi chosen for such studies were the common soil inhabitants *Mortierella isabellina* (Oudemans), *Mortierella pusilla* (Oudemans) and *Trichoderma viride* (Persoon) and the aquatic species *Saprolegnia parasitica* (Coker). Justifications for this choice of organisms are now briefly summarized. *M. isabellina* was chosen because

it has been found active in the degradation of other complex molecules (33) and *M.pusilla* would then provide a comparison between two species of the same genus. *T.viride* was chosen since it readily cleaves carbon-oxygen bonds in the production of glucose from cellulose (34), and also because it has been found capable of degrading several chlorinated hydrocarbon insecticides, including DDT (35). *S.parasitica* was used as it is primarily an ubiquitous aquatic fungus and would therefore be an excellent representative of aquatic organisms. However, before such biodegradation studies between fungi and methoxychlor can be undertaken, a logical requirement would be an initial assessment of any toxic effects of methoxychlor and its derivatives with such organisms.

The toxicity and metabolic studies were carried out in pure culture to avoid contamination by other unwanted organisms which could present difficulties by confusing the results. The toxicity investigation was an early part of this thesis and the results have been published elsewhere (36).

It is recognised that metabolic studies with fresh-water algae are an essential part of the environmental chemistry of methoxychlor. Such studies are not included in this thesis, which is not intended

to deal exhaustively with all aspects of methoxychlor chemistry.

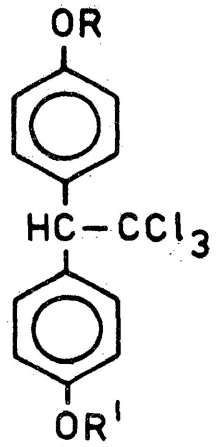
However, it is hoped that the results reported here will help to fill some of the present gaps in existing knowledge concerning this pesticide.

CHAPTER 2: PREPARATION, ANALYSIS AND SPECTROSCOPY OF METHOXYCHLOR DERIVATIVES

Preparations. Derivatives of methoxychlor are not commercially available. However, many of these compounds have been prepared by various workers and published methods have been used for the synthesis of these derivatives employed throughout this study.

Kapoor *et. al.* (1) have proposed a number of mono- and dihydroxy compounds which may result from metabolism of methoxychlor by *o* - dealkylation of one or both of the aromatic methoxy groups. Another alteration of methoxychlor may involve the central part of the molecule in a way similar to the dehydrochlorination or reductive dechlorination reactions observed for DDT (27). Thus, the compounds involved in these syntheses may be divided into three categories. The first of these consists of the diaryltrichloroethanes I to IV,* a second category includes the diaryldichloroethylenes V to VII, and a third group of compounds contains the diaryldichloroethanes VIII and IX. All these structures are given on p.24. The

* Structural formulae in this chapter have been numbered differently from those in Chapter 1 and present numbers have been retained throughout the remainder of this thesis.

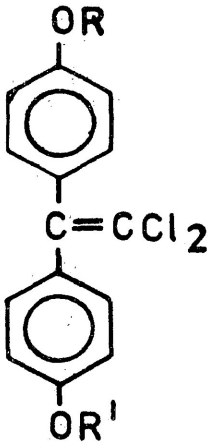


I Methoxychlor, $R = R' = CH_3$

II Ethoxychlor [2,2-bis (*p* - ethoxyphenyl)-1,1,1-trichloroethane], $R = R' = C_2H_5$

III Hydroxychlor [2,2-bis (*p* - hydroxyphenyl)-1,1,1-trichloroethane], $R = R' = H$

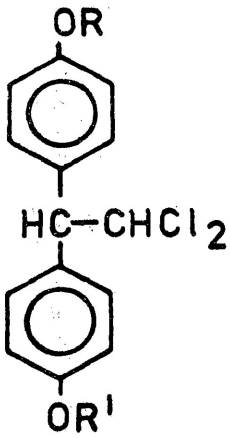
VI Methoxyhydroxychlor [2-*p*-methoxyphenyl-2-*p* - hydroxyphenyl-1,1,1- trichloroethane], $R = CH_3$, $R' = H$



V Methoxychlor olefin [2,2-bis (*p* - methoxyphenyl) -1,1- dichloroethylene], $R = R' = CH_3$

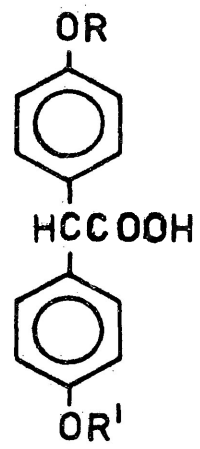
VI Hydroxychlor olefin [2,2-bis (*p* - hydroxyphenyl) -1,1- dichloroethylene], $R = R' = H$

VII Methoxyhydroxychlor olefin [2-*p*-methoxyphenyl-2-*p* - hydroxyphenyl-1,1- dichloroethylene], $R = CH_3$, $R' = H$



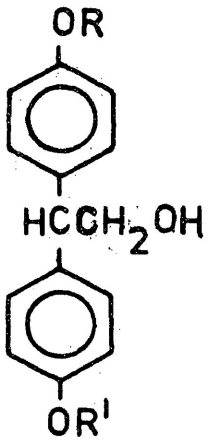
VIII Methoxydichloromethyl derivative [2,2-bis (*p* - methoxyphenyl)-1,1- dichloroethane], $R = R' = CH_3$

IX Hydroxydichloromethyl derivative [2,2-bis (*p* - hydroxyphenyl)-1,1- dichloroethane], $R = R' = H$



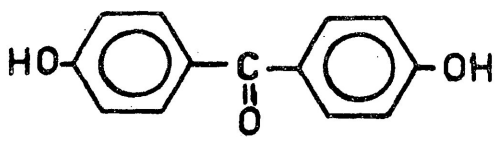
X 2,2-bis (*p* - methoxyphenyl) acetic acid, R = R' = CH_3

XI 2,2-bis (*p* - hydroxyphenyl) acetic acid, R = R' = H

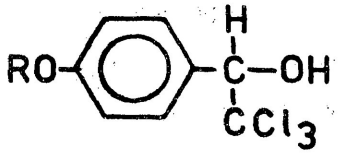


XII 2,2-bis (*p* - methoxyphenyl) ethanol, R = R' = CH_3

XIII 2,2-bis (*p* - hydroxyphenyl) ethanol, R = R' = H



XIV *p*,*p*'- Dihydroxybenzophenone



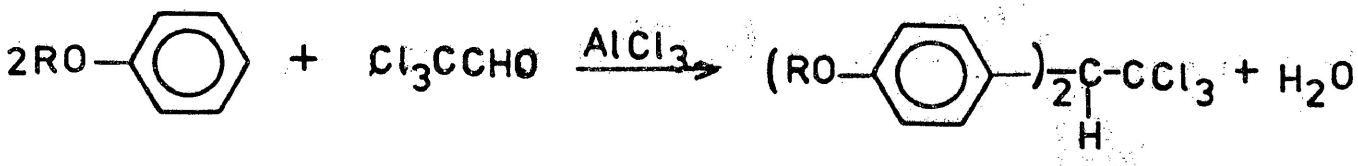
XV *p* - methoxyphenyl-1,1,1-trichloroethanol

acetic acids X and XI and the alcohols XII and XIII were not considered in the present study as these compounds have not been conclusively identified in biodegradation studies with methoxychlor to date. These structures are given on p.25.

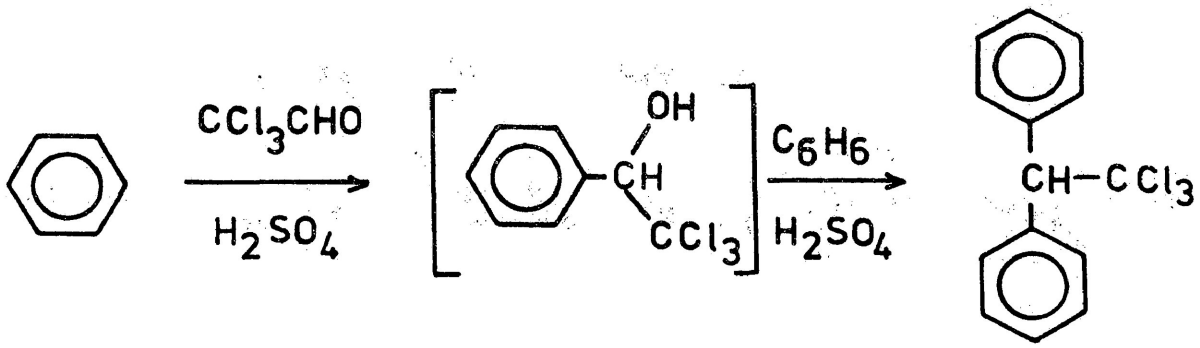
The formation of the diaryltrichloroethanes is analogous to that of the Baeyer condensation (37). The general reaction is represented by Eq.[1] (see p.27) and the reaction mechanism which takes place in two steps is shown in Scheme 1* (see p.28). The first step involves the nucleophilic addition of a substituted benzene to trichloroacetaldehyde (chloral) which yields a secondary alcohol. The second step is a Lewis acid-catalysed substitution of the hydroxyl group of that secondary alcohol by a second molecule of the substituted benzene, yielding a 1,1,1-trichloro-2,2-diarylethane with the loss of a molecule of water. This mechanism has been proposed by Frankforter and Kritchevsky (38). The diaryltrichloroethanes prepared by this method were 2,2-bis (*p* - hydroxyphenyl)-1,1,1- trichloroethane, (Hydroxychlor,III) and 2,2-bis (*p* - ethoxyphenyl)-1,1,1-trichloroethane, (Ethoxychlor,II). This ethoxy derivative

* Schemes have been numbered per chapter (i.e. numbers for schemes in this chapter may not necessarily be the same as those in other chapters.).

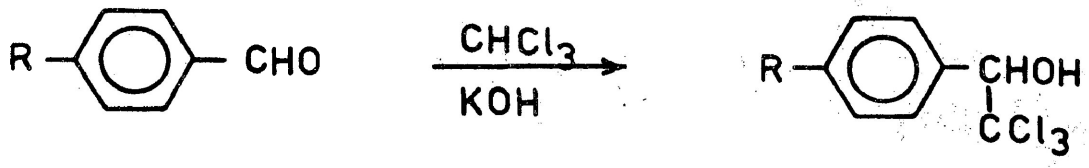
Eq.[1] FRANKFORTER & KRITCHEVSKY , 1935.



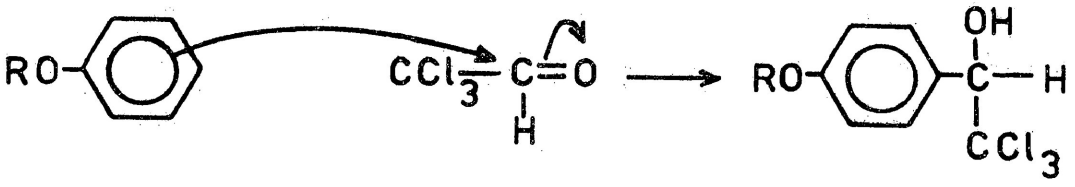
Eq.[2] CHATTAWAY & MUIR , 1934 BAYER , 1872.



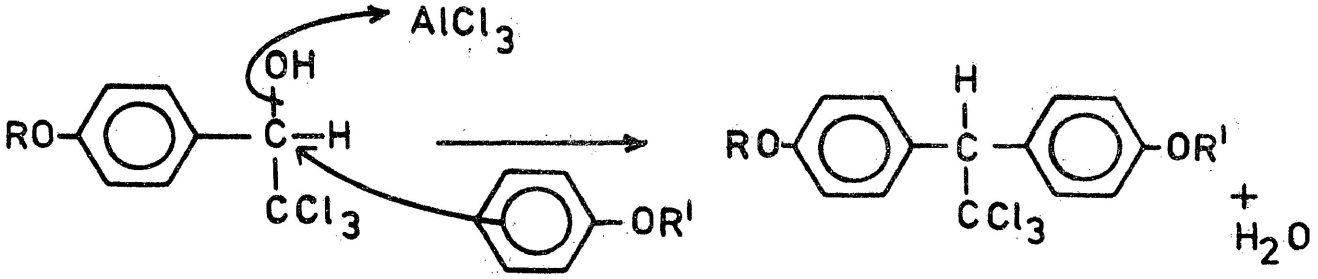
Eq.[3] HOWARD , 1935.



STEP 1



STEP 2



Scheme 1. Reaction mechanism for the formation of the diaryltrichloroethanes.

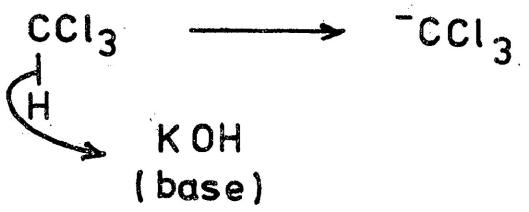
was prepared for use as an internal reference in gas chromatographic analyses (see p.44). A laboratory synthesis of methoxychlor,(I) would follow the same mechanism (Scheme 1).

To prepare the unsymmetrically substituted compounds such as methoxyhydroxychlor,(IV) the intermediate alcohol, (XV, p.25) must first be isolated. Theoretically, this could be achieved by using a one molar quantity of the substituted benzene (e.g. anisole) with respect to the amount of chloral, as indicated by Chattaway and Muir (39) who prepared the unsubstituted compound (Eq.[2] see p.27).

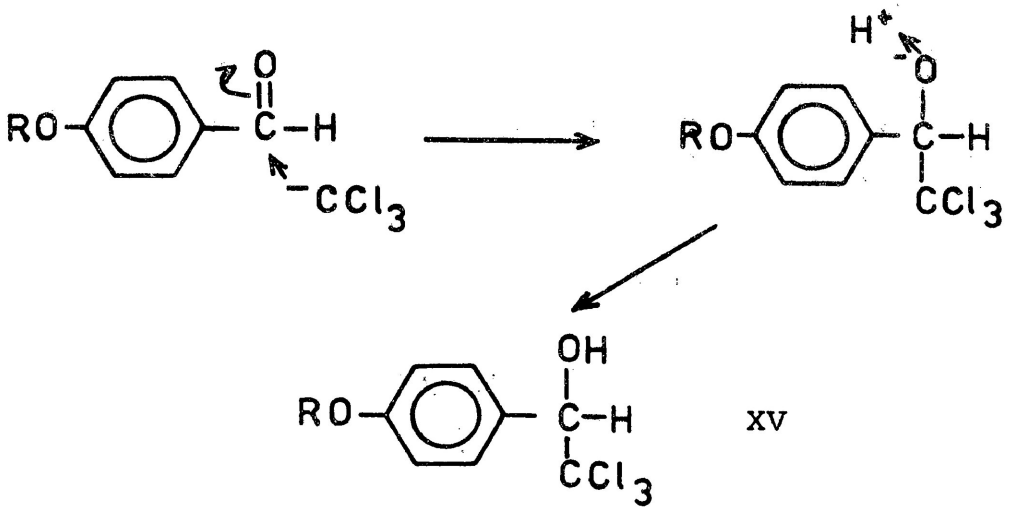
However, isolation of the intermediate alcohol proved to be difficult since such reactions usually proceed a step further to the diaryl substituted compound, which is then obtained in lower yield. A better preparation of the asymmetrically substituted compounds involves the preparation of the carbinol in such a way that the formation of the diaryl substituted compound is not possible. Thus, *p* - methoxyphenyl-1,1,1-trichloroethanol,(XV) was prepared by reacting chloroform with *p* - anisaldehyde, analogous to the method used to prepare *p* - chlorophenyl-1,1,1- trichloroethanol by Howard (40). This reaction is given in Eq.[3] (see p.27) and the mechanism is illustrated in Scheme 2 (see p.30). The first step is the abstraction of a proton from chloroform by the base, followed by the nucleophilic addition of the trichloromethyl-carbanion produced, to the aldehyde, resulting in the desired carbinol. Condensation of this alcohol with phenol according to the second step in Scheme 1 yields methoxyhydroxychlor, (IV).

The olefins V to VII (see p.24) were prepared by a conventional dehydrochlorination of the corresponding trichloroethanes. It is reasonable to assume a conventional E_2 elimination, shown in Scheme 3 (see p.31), which has been proposed by Cristol *et. al.* (41, 42).

STEP 1



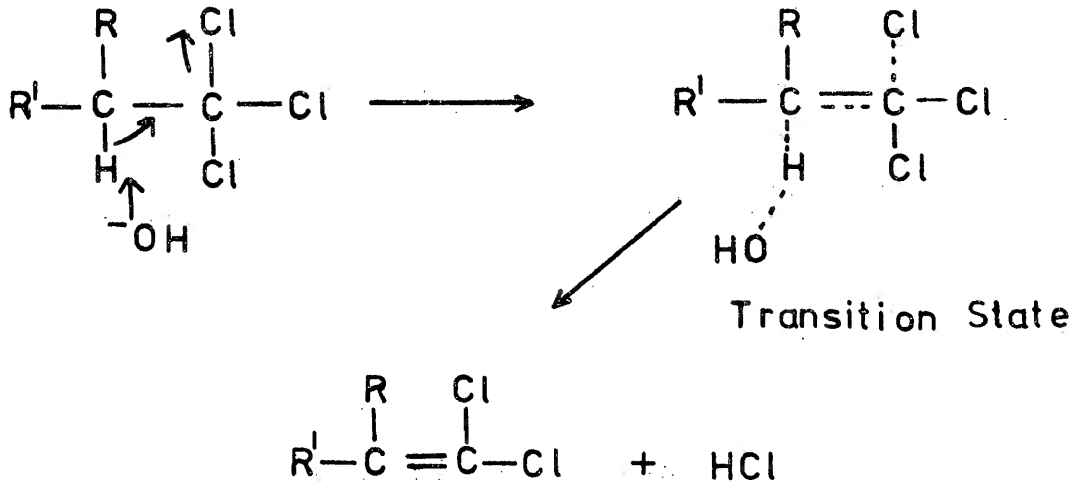
STEP 2



Scheme 2. Reaction mechanism for the formation of the *p* - methoxyphenyl-1,1,1- trichloroethanol,(XV).

There is no reason to expect a restriction of rotation about the relevant carbon-carbon bond, and moreover, the presence of the three chlorine atoms ensures that the required antiperiplanar conformation can be attained. This reaction is represented by Eq.[4] (see p.32). The rate of dehydrochlorination of DDT-type compounds to

form the corresponding ethylenes is controlled by the acidity of the α-hydrogen * and is therefore influenced by the electron withdrawing capacity of the *p* and *p'* substituents as measured by summation of Hammett σ values (41, 42). The olefins prepared for the present



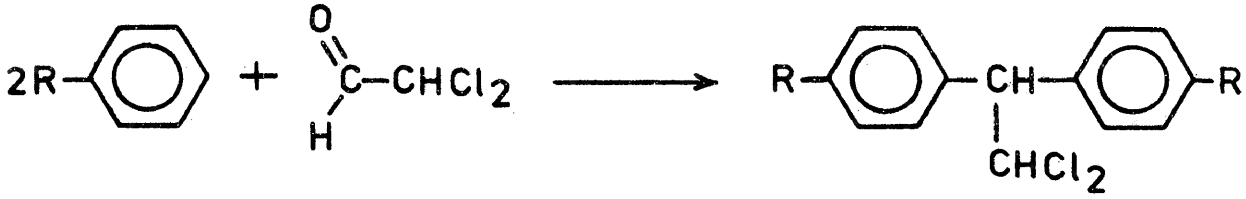
Scheme 3. Reaction mechanism for the formation of the diaryldichloroethylenes.

study had either methoxy or hydroxy *p,p'* substituents. Thus, since the hydroxy group is more strongly electron withdrawing than the methoxy group, the ease of formation of the olefins would be expected to be in the order of hydroxychlor olefin,(VI) > methoxyhydroxychlor olefin,(VII)>

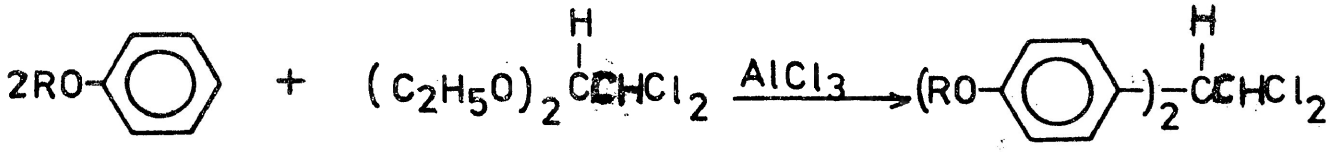
* The α-hydrogen refers to the hydrogen attached to the carbon that is adjacent to the carbon containing the trichloroethane group.

Eq.[4] CRISTOL et.al., 1952.

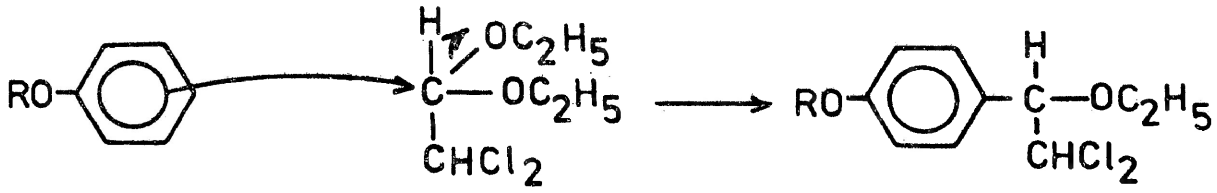
Eq.[5] SHIRLEY , 1950.



Eq.[6] CRISTOL et.al., 1952.

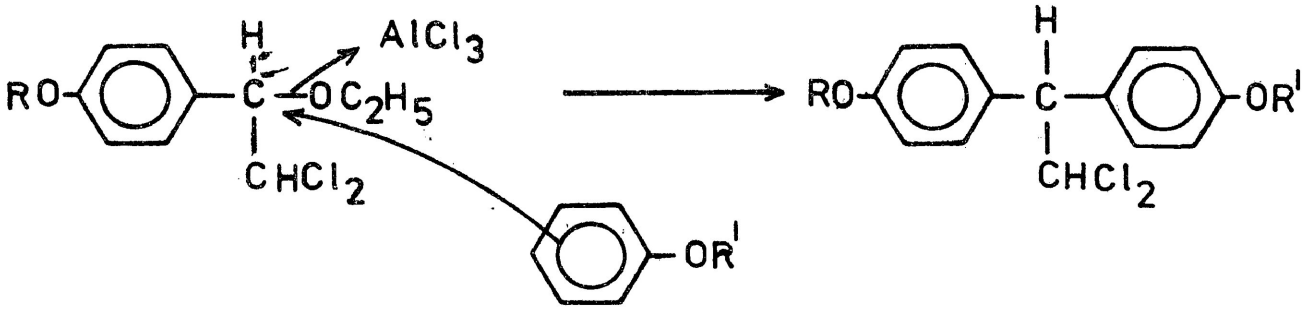


STEP 1



XVI

STEP 2



Scheme 4. Reaction mechanism for the formation of the diaryldichloroethanes.

> methoxychlor olefin,(V). However, no noticeable difference in yield was observed during the preparation of these compounds.

The diaryl substituted dichloroethanes VIII and IX(see p.24), may be prepared by the reaction of the suitably substituted benzene with dichloro-acetaldehyde

(43) shown in Eq.[5] or dichloro-diethylacetal (12) as represented by Eq.[6] (see p.32). The latter reaction was used and the reaction mechanism (Scheme 4, see p.33) is similar to that given for the trichloroethanes. Step 1 involves the nucleophilic addition of the substituted benzene to the acetal producing the intermediate,(XVI) (Scheme 4, see p.33). The second step is a Lewis acid-catalysed substitution of the ethoxy group of that intermediate,(XVI) by a second molecule of the substituted benzene, yielding a 1,1- dichloro-2,2- diarylethane. It is difficult to isolate the intermediate product,(XVI) and only the dimethoxy,(VIII) and dihydroxy,(IX) compounds were synthesized.

A brief discussion of the experimental details of these syntheses is warranted because it was found that the published methods do not fully disclose some of the difficulties that may be encountered in these preparations.

The published method for synthesizing the diaryl substituted trichloroethanes gave good results for ethoxychlor,(II). For hydroxychlor,(III) the preparation by Kapoor *et. al.* (1) was followed, but these authors did not provide experimental details. Initially, after the condensation reaction was complete (as represented by Scheme 1), attempts were made to isolate the product by extraction with chloroform and benzene. It was found

that the product was virtually insoluble in these solvents. The solvent of choice for this extraction was found to be ethyl acetate. The product, initially obtained as a viscous brown oil was successfully recrystallized from a benzene-ethanol mixture instead of methylene chloride with a trace of ethanol as recommended by the authors.

It should be reiterated that this insolubility of hydroxychlor,(III) in benzene could have important consequences in residue analyses involving these compounds, primarily because benzene is used as a solvent in many standardized extraction procedures normally used in residue analyses. One such difficulty with the recovery of hydroxychlor,(III) may have been involved in a study of the interaction of methoxychlor with the bacterium *K.pneumoniae* (19). These authors reported that much of the methoxychlor is lost during incubation. Since benzene was used in their extraction procedure it is probable that polar metabolites such as hydroxychlor were not recovered during that investigation. This provided the impetus for reexamining the work of Mendel *et. al.* (19) and the results of that reexamination have become a major part of this thesis (see Chapter 3).

The mixed compound, methoxyhydroxychlor,(IV) was somewhat more difficult to prepare since the synthesis

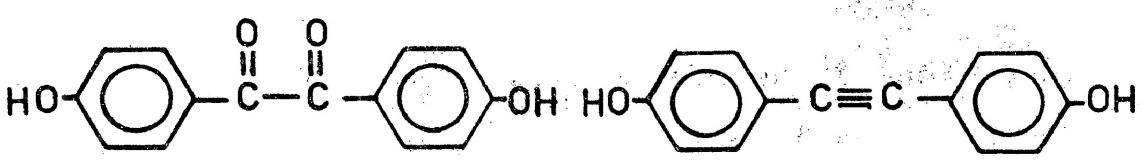
involved the isolation of the intermediate alcohol,(XV) and different methods have been published for the preparation and isolation of such intermediates. Schneller and Smith (37) reported that the condensation of benzene or its *p* - substituted derivatives with chloral, using anhydrous aluminium trichloride, can be controlled so as to produce the carbinol, the diarylethane or both, depending on the nature of the substituent on the benzene ring and the reaction conditions. These authors found that with anisole, ninety percent (90%) of the product was the diarylethane. Frankforter and Kritchevsky (38) have also used this method to prepare methoxychlor. An alternative preparation for the carbinol has also been reported by Chattaway and Muir (39) (Eq.[2] see p.27). They used the Baeyer sulphuric acid condensation of chloral with benzene derivatives. These authors found that by reversing the customary order of mixing the reagents, i.e. adding the benzene derivative to an excess of chloral, good yields of the carbinol could be obtained. Both of the preceding methods were attempted in the present work but in both cases the diaryl compounds were the only products which could be isolated. Therefore the carbinol was prepared by the method of Howard (40) according to Eq.[3] (see p.27) and Scheme 2 (see p.30), using *p* - anisaldehyde instead of *p* - chlorobenzaldehyde.

This gave acceptable yields of the carbinol and the further reaction of this compound with phenol according to Schneller and Smith (37) gave the desired asymmetrical compound,(IV). For the recrystallization of the final product a chloroform-cyclohexane mixture was found to be superior to the benzene-cyclohexane mixture used by Kapoor *et. al.* (1).

The yields obtained for hydroxychlor,(III) and methoxyhydroxychlor,(IV) were only moderate. A possible explanation for this is the fact that the corresponding olefin may be produced simultaneously. Fleck and Haller (44) have used anhydrous aluminium trichloride as a catalyst for the elimination of HCl from such *p* - substituted trichloroethanes to produce the corresponding olefins.

The preparation of methoxychlor olefin,(V) was carried out according to Kapoor *et. al.* (1). The reaction, which involves refluxing methoxychlor with 50% ethanolic potassium hydroxide, gave good yields and no complications were encountered. However, for the preparation of hydroxychlor olefin,(VI) and the methoxyhydroxychlor olefin,(VII), the method of Hubacher (45) gave better results. Thus, quantities of hydroxychlor,(III) and methoxyhydroxychlor,(IV) were each refluxed for 0.5hr. with 3N methanolic potassium hydroxide, resulting in the

formation of the corresponding olefins. Due to the high solubility of hydroxychlor olefin,(VI) in ethanol, this solvent was found unsuitable for the recrystallization of this compound as suggested by Kapoor *et. al.*(1), 20% aqueous ethanol was found to give better results. The low yield of hydroxychlor olefin,(VI) when Kapoor's method (1) was used for the elimination reaction may have been due to the formation of 4,4' - dihydroxybenzil, (XVII)and 4,4' - dihydroxytolan,(XVIII). Hubacher (45) has used a similar method to prepare these compounds. This author used aqueous potassium hydroxide instead of 50% ethanolic potassium hydroxide. Therefore the formation of such compounds as the substituted benzils and tolans could account for the lower yields in preparing these other olefins [i.e. hydroxychlor olefin, (VI) and methoxyhydroxychlor olefin,(VII)].



XVII 4,4' - dihydroxybenzil

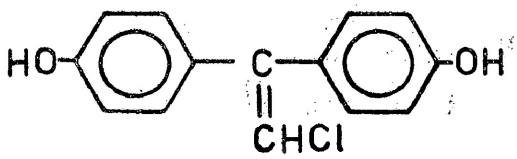
XVIII 4,4' - dihydroxytolan

Preparation of the diaryldichloroethanes VIII and IX was carried out according to Zepp *et. al.* (12) who used this method to prepare the dimethoxydichloroethane,(VIII). It follows from the mechanism of the reaction (Scheme 4,see p.33) that a small excess of aluminium trichloride is desirable to ensure a quantitative reaction of the starting materials. The dimethoxy derivative was initially obtained as a light brown oil, which on solidification, could be conveniently recrystallized from a benzene-ethanol mixture. The dihydroxydichloro compound,(IX) was also prepared by this method using liquid phenol instead of anisole. This reaction led to the desired product without difficulties. As far as can be ascertained the preparation of compound IX has not been reported in the literature before.

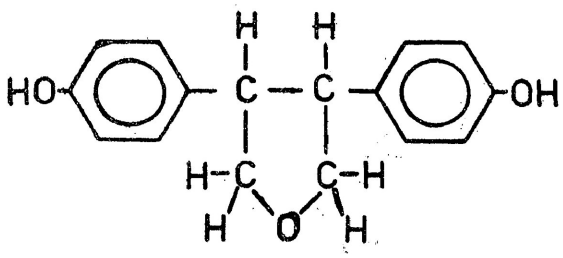
Low yields of these dichloro compounds were obtained, and a number of unidentified byproducts were evident in the reaction mixture. A number of attempts were made to improve the yields in these reactions, e.g. by varying the reaction temperature. These attempts were unsuccessful, but were not pursued, since only small quantities of these compounds were required.

An attempt was made to prepare the hydroxymonochloro ethylene,(XIX) by an elimination of hydrogen chloride

from the corresponding dichloro compound. The treatment of the dihydroxydichloro compound,(IX) with alkali, however led to a product $C_{16}H_{16}O_3$, as established by both elemental analysis and a precise mass measurement on the molecular ion. The NMR spectrum is complicated and an unequivocal assignment of the proton signals was not possible. Structure (XX) would accommodate these data, but could not be confirmed on the small quantity of material available.



XIX Hydroxymonochloro ethylene[2,2-bis (*p* - hydroxyphenyl) -1- monochloroethylene]



XX Proposed structure for product $C_{16}H_{16}O_3$

Such a structure is not unreasonable in view of the fact that rearrangements, similar to the benzilic acid rearrangement, can occur in this type of reaction as mentioned by Hubacher (45) (see p.38). These transformations may not take place in an environmental situation in view of the reaction conditions used in this case, but the reaction itself does generate an interesting synthetic problem. It is recommended that this reaction type be further studied, not from a viewpoint of methoxychlor degradation but as an exercise in the elucidation of the various pathways that may be involved in such reactions leading to the formation of different products. The anomalous product obtained when the preparation of the hydroxymonochloro-ethylene,(XIX) was attempted is in part the justification for looking at NMR and mass spectral data in more detail.

Analyses. For preliminary identifications of these various methoxychlor derivatives a thin layer chromatography (TLC) system described by Kapoor *et. al.* (1) was used as a routine method in the present study. These authors reported that the different methoxychlor derivatives undergo different chromogenic reactions with a spray reagent containing 0.5% diphenylamine and 0.5% zinc chloride in acetone. The colours developed after the plates were sprayed with the reagent, heated for ten minutes at 110°

and then exposed to ultraviolet light (254mμ) for at least five minutes. The diaryltrichloroethanes produced grey or black colours, the diaryldichloroethylenes were pink and the diaryldichloroethanes gave bright blue spots.

Gas chromatographic techniques for analysing methoxychlor and its breakdown products have been, to date, primarily concerned with the parent compound and in some instances with the olefin,(V) and the dichloromethyl derivative,(VIII).

Mendel *et. al.* (19) have used gas chromatography qualitatively to identify methoxychlor,(I), methoxychlor olefin,(V) and the dimethoxydichloromethyl derivative, (VIII), and used a quantitative method only for determining methoxychlor itself.

Castro and Yoshida (17) studied the degradation of this pesticide in flooded soils in the Philippines, but only the residual methoxychlor was monitored quantitatively by gas chromatography.

MacNeil *et. al.* (11) investigating the photolysis of methoxychlor, used gas chromatography for the identification of the two major photodecomposition products, methoxychlor olefin,(V) and the dichloromethyl derivative,(VIII).

Thompson *et. al.* (46) determined the relative retention ratios of ninety-five pesticides and metabolites on nine gas liquid chromatographic columns over a temperature

range of 170-204° in the electron capture and flame photometric (FPD) modes. Methoxychlor, but none of its known derivatives was included in that report.

Wolfe *et. al.* (47) studied the methoxychlor hydrolytic degradation pathway in water at pH's common in the aquatic environment and employed both electron capture and flame ionization detectors in the identification of breakdown products.

There are other examples (31,32) cited in the literature where gas liquid chromatography is used in investigations concerning methoxychlor, but none deal with phenolic methoxychlor derivatives and furthermore the majority are of a qualitative nature. It was found that the phenolic derivatives of methoxychlor, which were used in the present study, could not be eluted from gas chromatographic columns containing conventional stationary phases. It was therefore necessary to develop a method that would allow both the qualitative and quantitative analyses of such compounds. Stationary phases chosen for initial study were the silicone gum rubber (SE-30), which has been used alone and mixed with other stationary phases in pesticide analyses (11,17,31), Dexsil 300, OV 17/OV101 and SE-30/OV210. Column temperatures of 220-250° were found suitable for columns containing these packings. Also

a simple silylation procedure proved to be sufficient for the analysis of all phenolic compounds in the methoxychlor series. This technique involved the introduction of the trimethylsilyl group [$-Si(CH_3)_3$] into a molecule in substitution for active hydrogens. This reduces the polarity of the compound and greatly increases the volatility. The reaction is quantitative, fast and is done at room temperature. Although some of the compounds had similar or identical retention times on one single column, the use of different column packings allowed a complete resolution as illustrated by the retention times relative to DDT as recorded in Table 1 (see p.45).

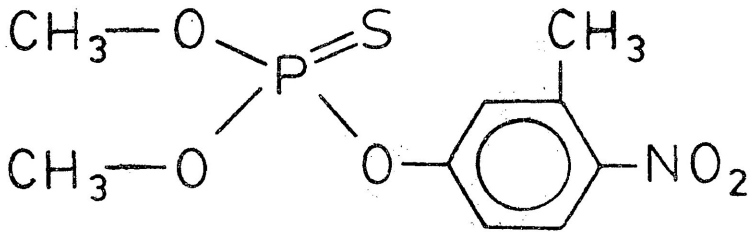
For quantitative measurements it was decided to use a method based on an internal standard, such as used by Takimoto *et. al.* (48) for fenitrothion,(XXI) and its derivatives. Both DDT and ethoxychlor,(II) were used as internal standards. Thus, from a series of samples containing varying quantities of the test compound, and all containing the same amount of internal standard, calibration curves were obtained, which then allowed the precise analysis of unknown samples, made up to standard volume with the same solution of the internal standard.

Table 1. Relative Retention Times (RRT) with respect to DDT of methoxychlor and its derivatives.

		Relative Retention Time (RRT, DDT=1)[b]			
	Column	SE-30(5%)	DEXSIL 300(3%)	OV17(3%)/ OV101(3%)	SE-30(4%)/ OV210(6%)
No.[a]	Compound				
I	Methoxychlor	1.23	1.35	1.63	1.29
V	Methoxychlor olefin	0.87	0.88	1.04	0.88
VIII	Methoxydichloromethyl	1.04	1.08	1.25	1.03
IV	Methoxyhydroxychlor	1.48	1.44	1.59	1.47
VII	Methoxyhydroxychlor olefin	1.04	0.89	1.04	1.03
III	Hydroxychlor	1.78	1.44	1.54	1.56
VI	Hydroxychlor olefin	1.30	0.99	1.04	1.12
IX	Hydroxydichloromethyl	1.44	1.22	1.25	1.32
XIV	Benzophenone	1.20	1.12	1.02	1.38

[a] The Roman numerals in parentheses refer to the structural formulae of methoxychlor and its derivatives shown on p.24 .

[b] Retention Times (min.) of DDT on the above columns are: SE-30(5%) = 5.75min.; Dexsil 300(3%) = 4.45min.; OV17(3%)/OV101(3%) = 4.80min.; SE-30(4%)/OV210(6%) = 3.4min..



Fenitrothion, (XXI) - [O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate]

Finally, it may be noted that this method, which allows the analysis of amounts down to 0.5µg. with a FID detector, may well be modified, for example by using more sensitive detectors (e.g. electron capture), to allow the trace analysis of these phenolic compounds as required in conventional residue analysis.

Spectroscopic Data. Information on nuclear magnetic resonance (NMR) spectroscopy of methoxychlor and its derivatives is scarce. Kapoor *et. al.* (1) have reported the τ-values of the proton signals for methoxyhydroxychlor, (IV) and its olefin, (VII). There are other reports that mention the use of NMR in the identification and characterization of some methoxychlor metabolites, but specific information is not provided. It was found that

characterization of some of the synthesized compounds was greatly facilitated when complete NMR spectral information was obtained for all compounds in this series as part of the present study. An example of the need for spectroscopic information is the anomalous result of the attempted synthesis of the monochloro-olefin,(XIX) described above (see p.39).

The spectra of the diaryltrichloroethanes I, III and IV are all distinctive, and the signals of the different protons are well separated. Ethoxychlor,(II) is easily identified by the characteristic signals arising from the ethoxy groups. In the spectra of methoxychlor, (I) and methoxyhydroxychlor,(IV),the methoxy groups are represented by signals at τ6.26 and τ6.20ppm respectively. The hydroxy group in the latter compound and in hydroxychlor, (III) are usually recognizable after deuterium exchange with deuterium oxide (D_2O). The typical doublet of doublets representing the eight aromatic protons of these compounds is found at τ2.3-3.4ppm. The α-proton signal is found at τ5.03 for methoxychlor,(I) and at τ4.85ppm for both methoxyhydroxychlor,(IV) and hydroxychlor,(III). The τ-values for these signals of the different protons in these trichloroethanes have been tabulated in Table 2 (see p.48).

Table 2. Nuclear magnetic spectral data of methoxychlor and its derivatives.

No.[a]	Compound	τ - values (ppm)				
		$-CHCl_2$	α - H	$-OCH_3$	-OH	Aromatic (8H)
I	Methoxychlor	-	5.03	6.26	-	2.4-3.4
III	Hydroxychlor	-	4.85	-	7.1	2.3-3.2
IV	Methoxyhydroxychlor	-	4.85	6.20	7.05	2.3-3.2
V	Methoxychlor olefin	-	-	6.25	-	2.7-3.25
VI	Hydroxychlor olefin	-		-	7.02	2.8-3.3
VII	Methoxyhydroxychlor olefin			6.17	7.15	2.6-3.3
VIII	Methoxydichloromethyl	5.2	7.0	7.7	-	4.2-4.75
IX	Hydroxydichloromethyl	3.16	6.5	-	7.01	2.6-3.35

[a] Roman numerals represent methoxychlor and its derivatives given on p.24.

The NMR spectra for the diaryldichloroethylenes V, VI and VII are easily distinquished from the trichloroethanes by the absence of the signal for the α-proton. Furthermore, the signals for the aryl subtituents can be used to distinquish between the three olefins and the results are also included in Table 2.

The NMR spectra for the diaryldichloroethanes VIII and IX quite clearly allow the identification of these compounds as distinct from the trichloroethanes. For the dimethoxydichloromethyl derivative, (VIII), both the α-proton signal and dichloromethyl ($-CHCl_2$) signal are doublets centred at τ7.0 and τ5.2ppm respectively. There is of course, no such splitting pattern present in the spectra of the corresponding trichloroethanes. In the spectrum of the dihydroxydichloromethyl derivative,(IX) the signal of the α-proton appears as a doublet centred at τ6.5ppm. The signal for the proton of the dichloromethyl group is obscured by that of the aromatic protons but was clearly observed at τ3.16ppm after spin decoupling, effected by irradiation of the α-proton. When the signal of the dichloromethyl proton was similarly irradiated, the signal of the α-proton collapsed to a singlet centred at τ7.0ppm. The proton signals for these dichloromethyl compounds have also been given in Table 2.

The NMR spectra for 4,4' - dihydroxybenzophenone,(XIV) is simple and the broad hydroxy signal (2H) is easily identified at τ6.9ppm.

Although this collection of NMR data, which has not been published before, did not shed any light on the structure of the unknown product described on p. 40 [i.e. the dehydrohalogenation of the dihydroxydichloro compound, (IX)], it is clear that this spectroscopic information will provide a useful background for any further work on the organic chemistry of these compounds.

Although the mass spectra of methoxychlor (49) and of its olefin,(V) and dichloromethyl derivative,(VIII) (11) have been reported, there is no systematic mass spectral study of the complete series in the literature.

Such a study was undertaken with the presently available compounds and it was found that mass spectra are clearly characteristic and can be interpreted without difficulty. A number of these spectra have been drawn and are illustrated in Figures 1 to 8. All compounds gave rise to clearly observable molecular ions and some similarities in fragmentation patterns were observed. Thus, it was found that for all the trichloro- and dichloroethanes, the base peaks were formed by the loss of the trichloro and dichloromethyl groups respectively and these fragmentation reactions are shown in Schemes 5

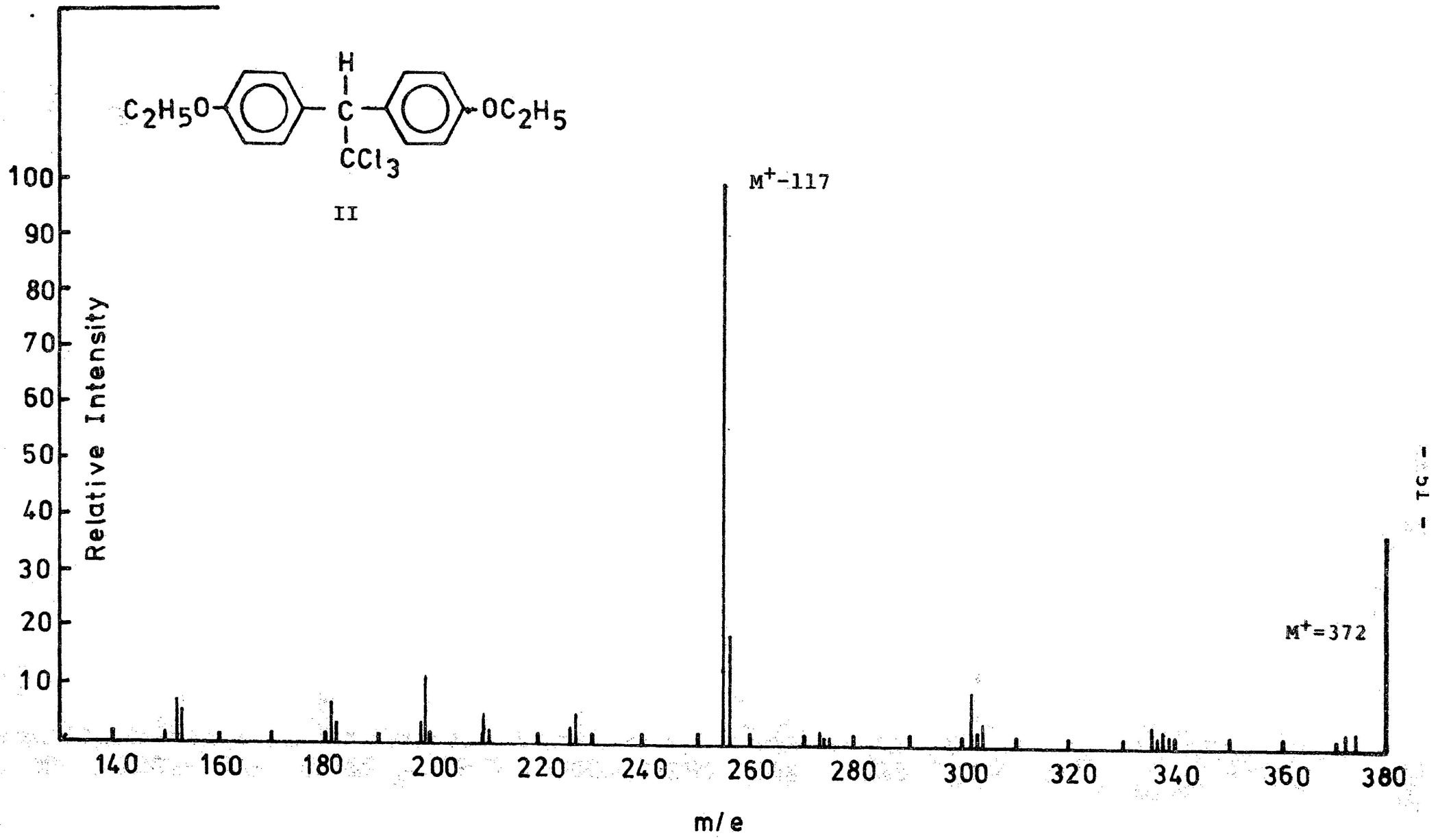


FIG. 1 Mass Spectrum of 2,2-bis (*p* - ethoxyphenyl)-1,1,1- trichloroethane, (Ethoxychlor, II).

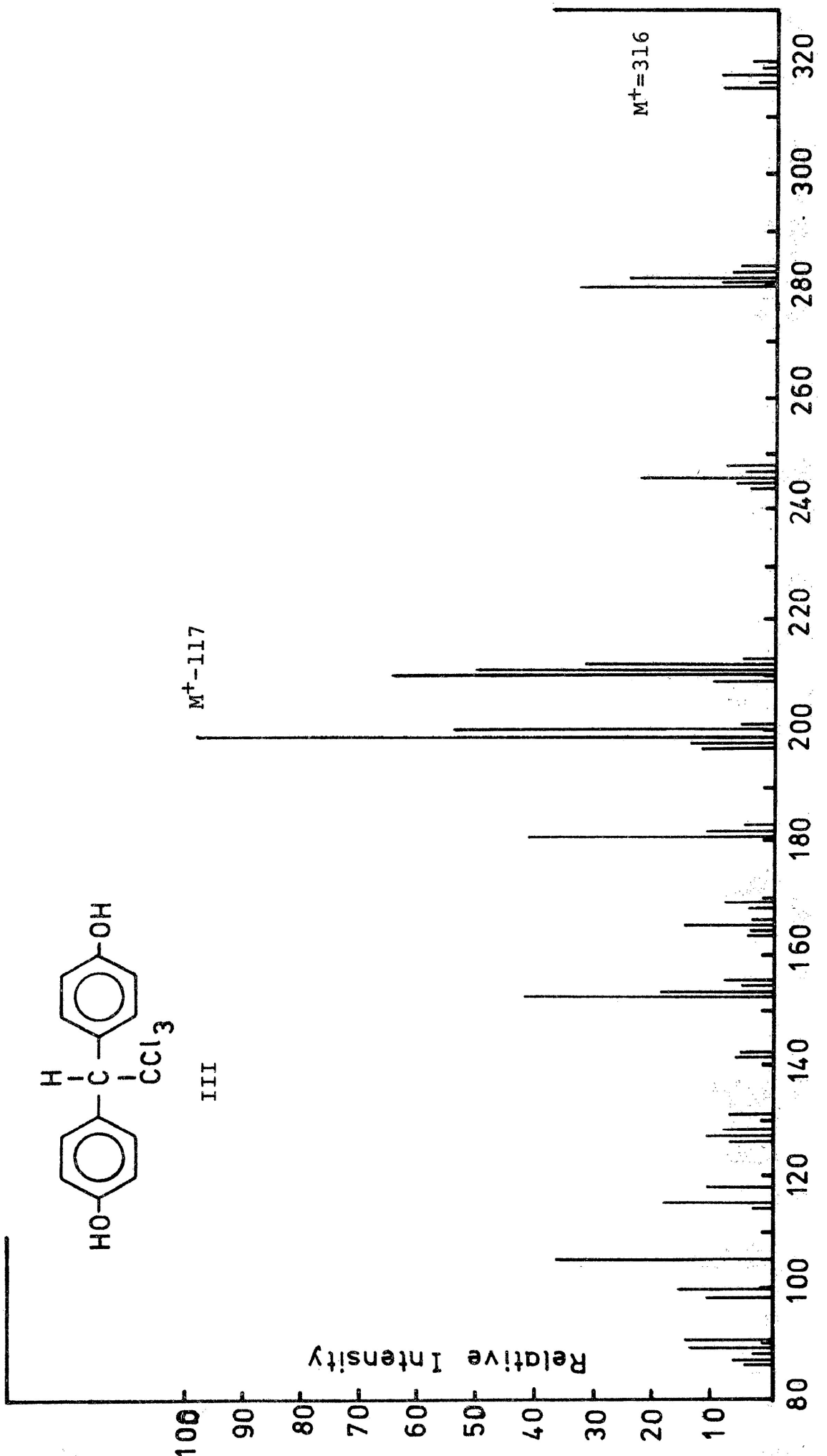


FIG. 2 Mass Spectrum of 2,2-bis (*p* - hydroxyphenyl)-1,1,1- trichloroethane, (Hydroxychlor, III).

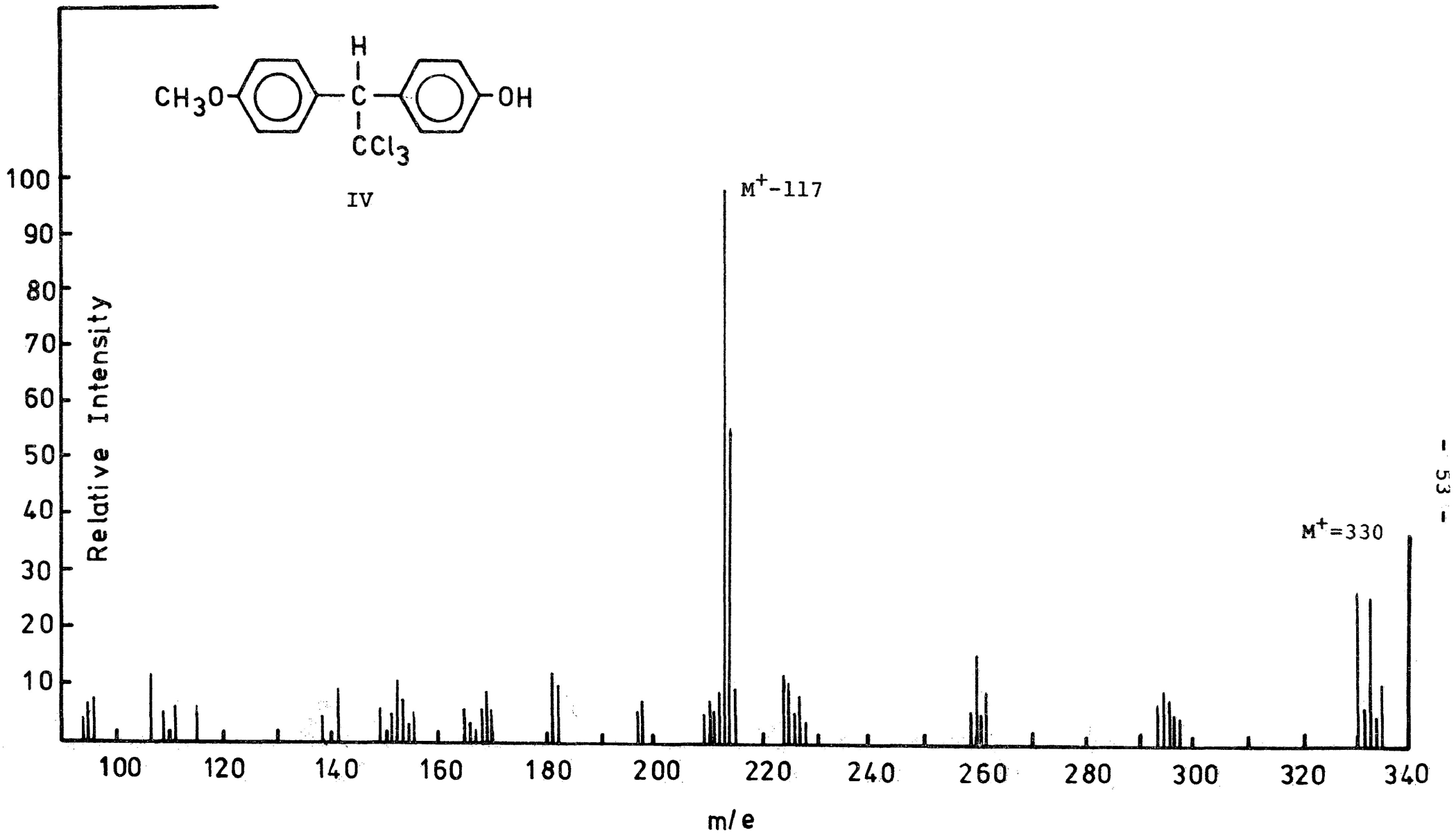


FIG. 3 Mass Spectrum of 2-*p*-methoxyphenyl-2-*p*-hydroxyphenyl-1,1,1- trichloroethane, (Methoxyhydroxychlor, IV).

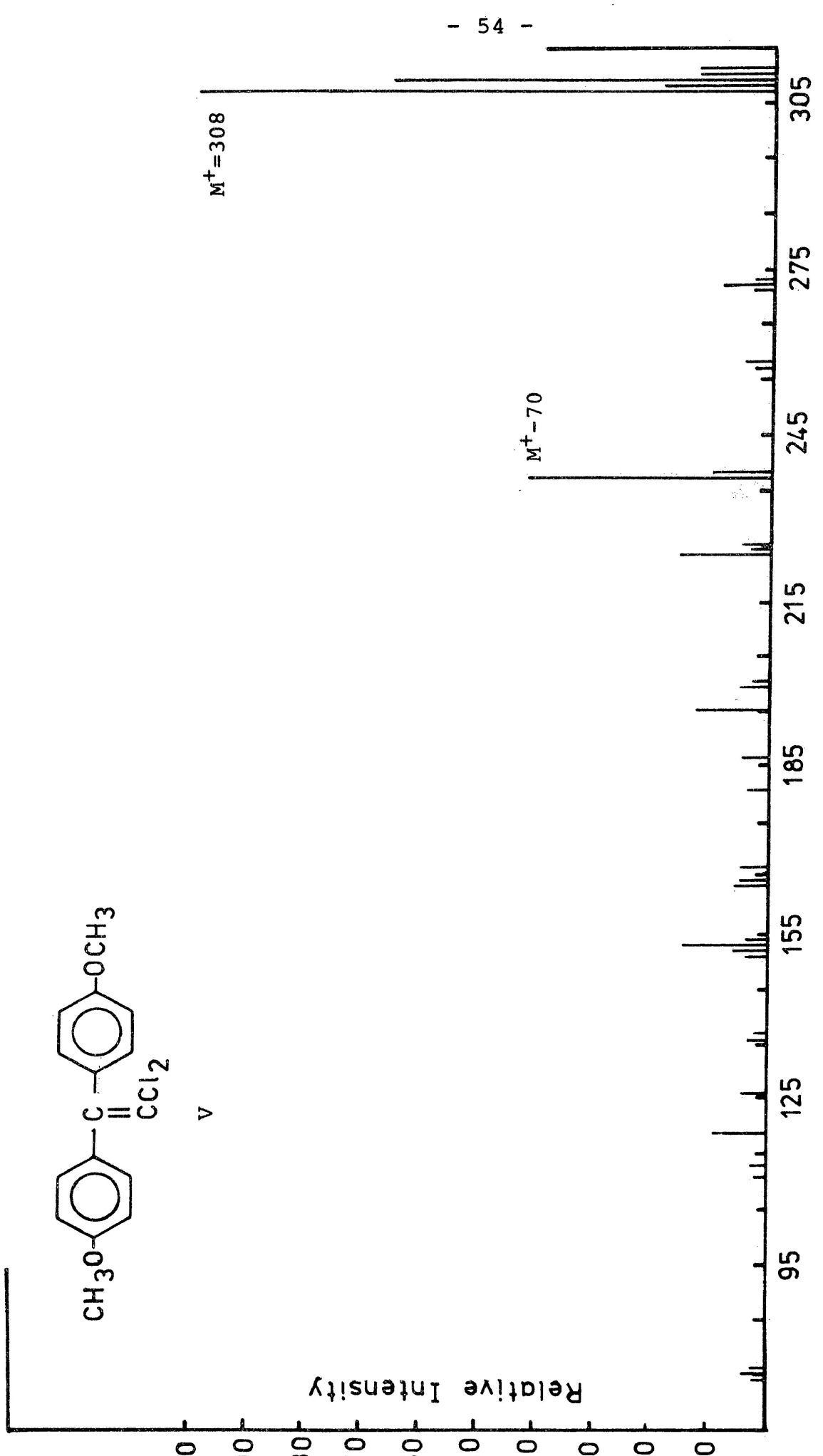


FIG. 4 Mass Spectrum of 2,2-bis (*p* - methoxyphenyl)-1,1- dichloroethylene, (Methoxychlor olefin,V).

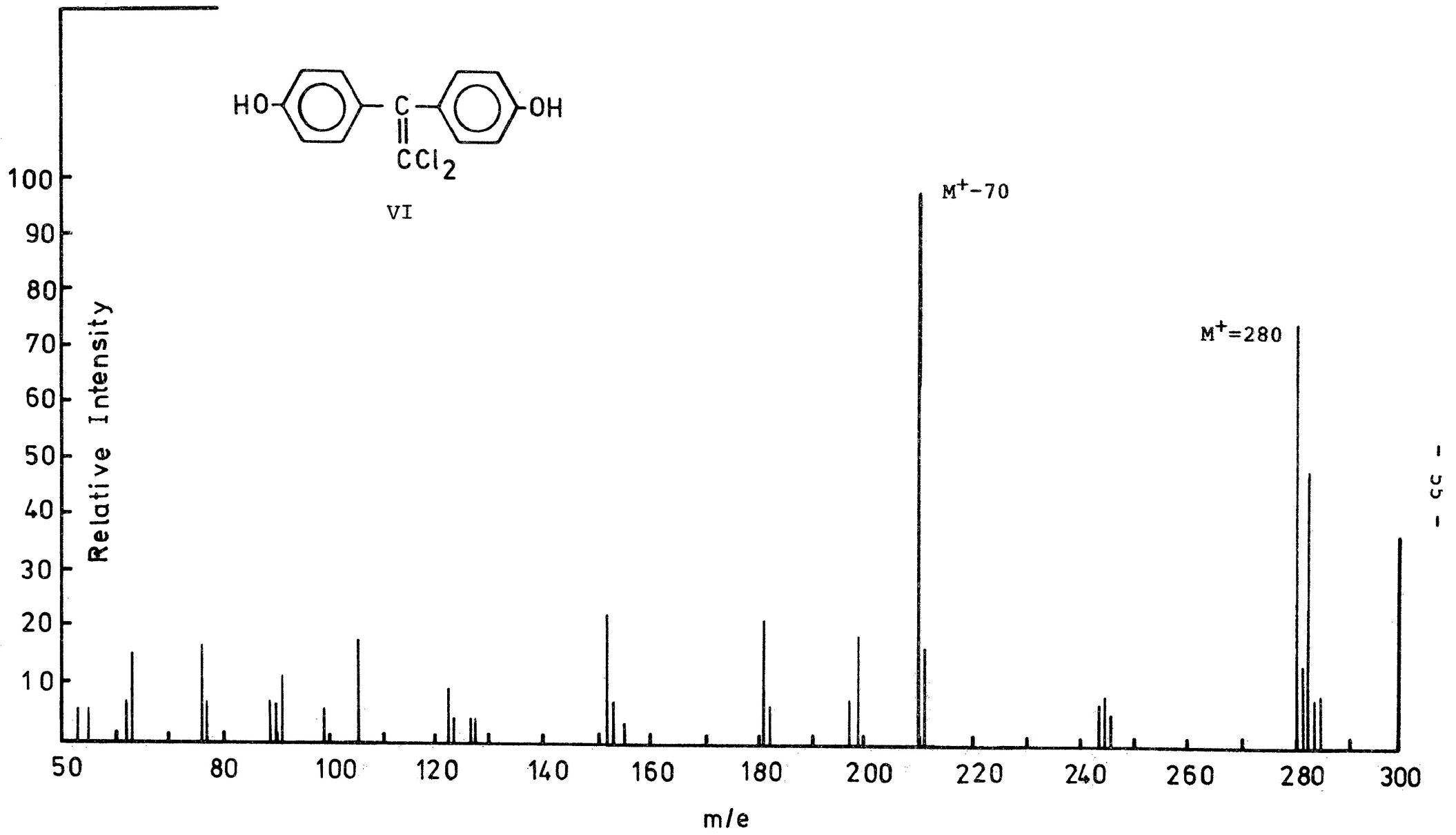


FIG. 5 Mass Spectrum of 2,2-bis (*p* - hydroxyphenyl)-1,1- dichloroethylene, (Hydroxychlor olefin, VI).

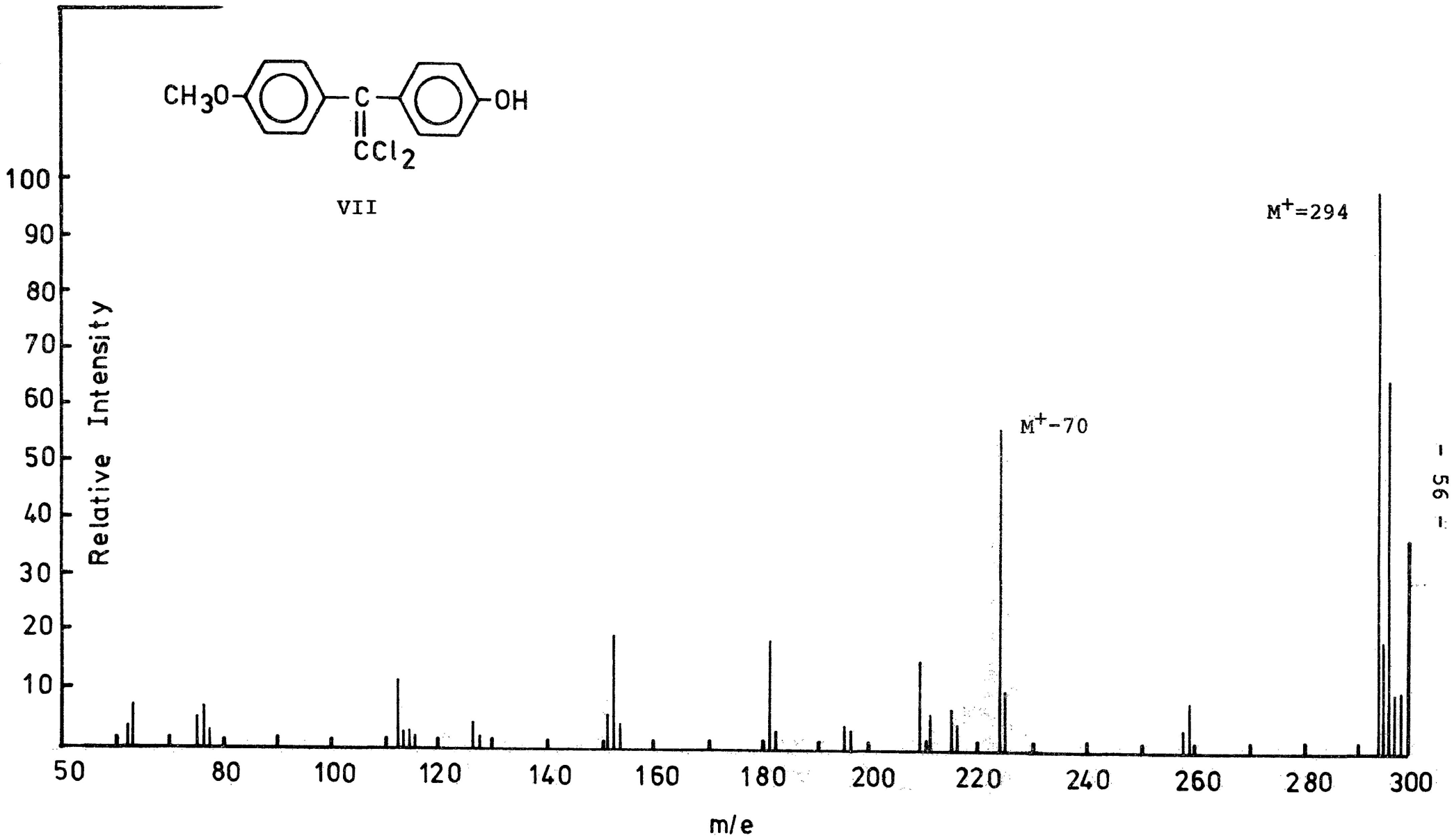


FIG. 6 Mass Spectrum of 2-*p*-methoxyphenyl-2-*p*-hydroxyphenyl-1,1- dichloroethylene, (Methoxyhydroxychlor olefin,VII).

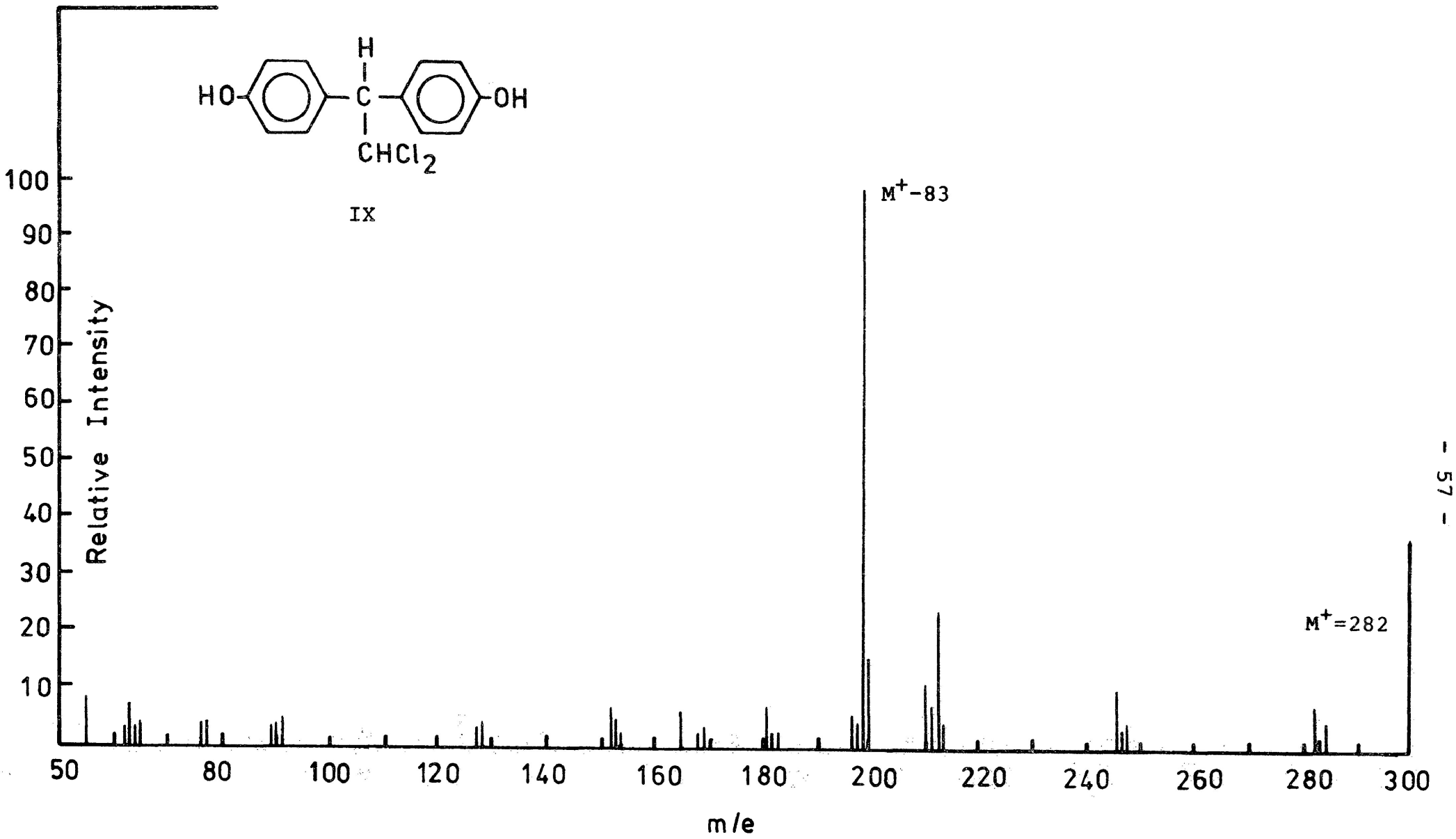


FIG. 7 Mass Spectrum of 2,2-bis (*p* - hydroxyphenyl)-1,1- dichloroethane, (Hydroxydichloromethyl derivative, IX).

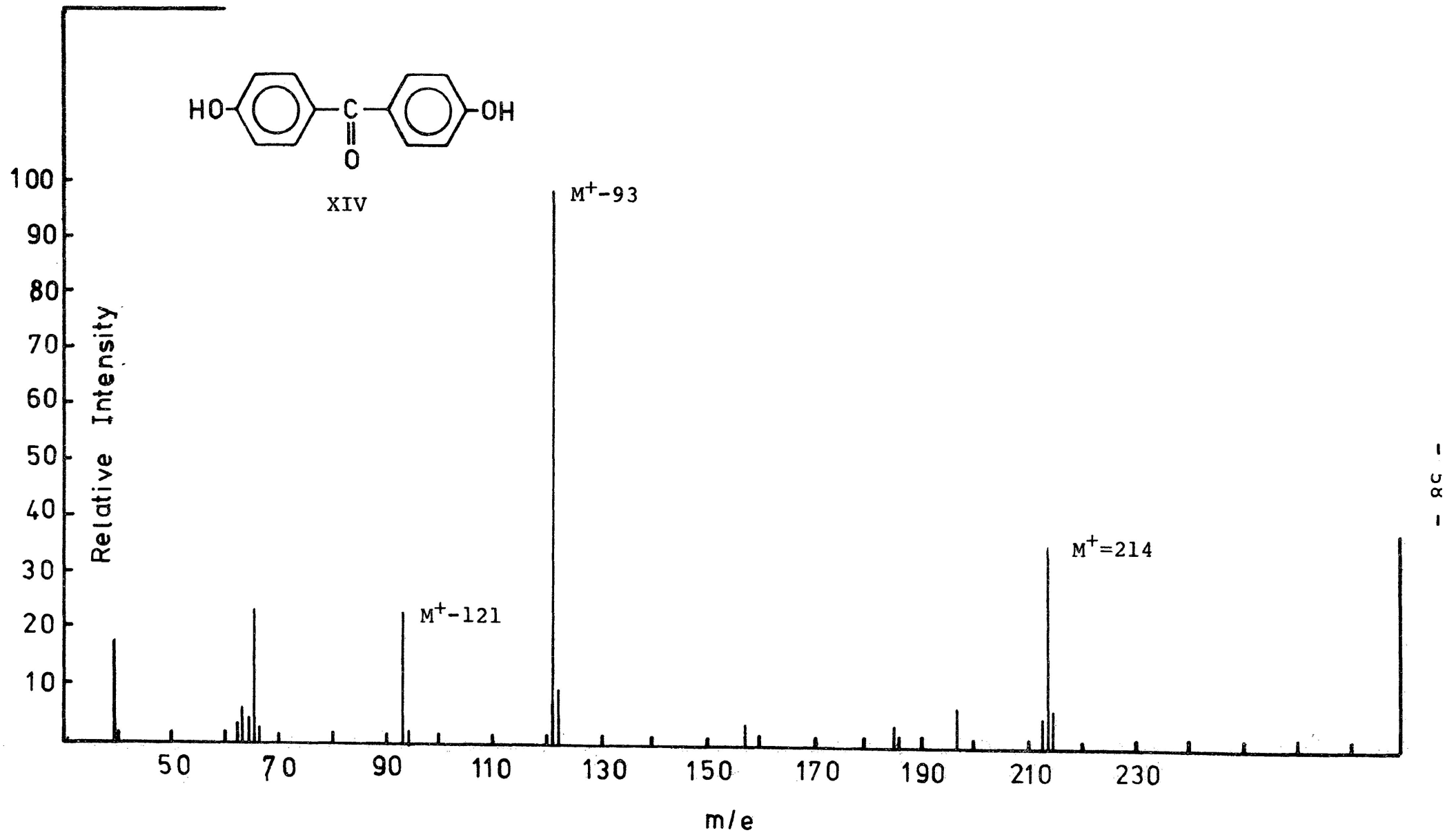
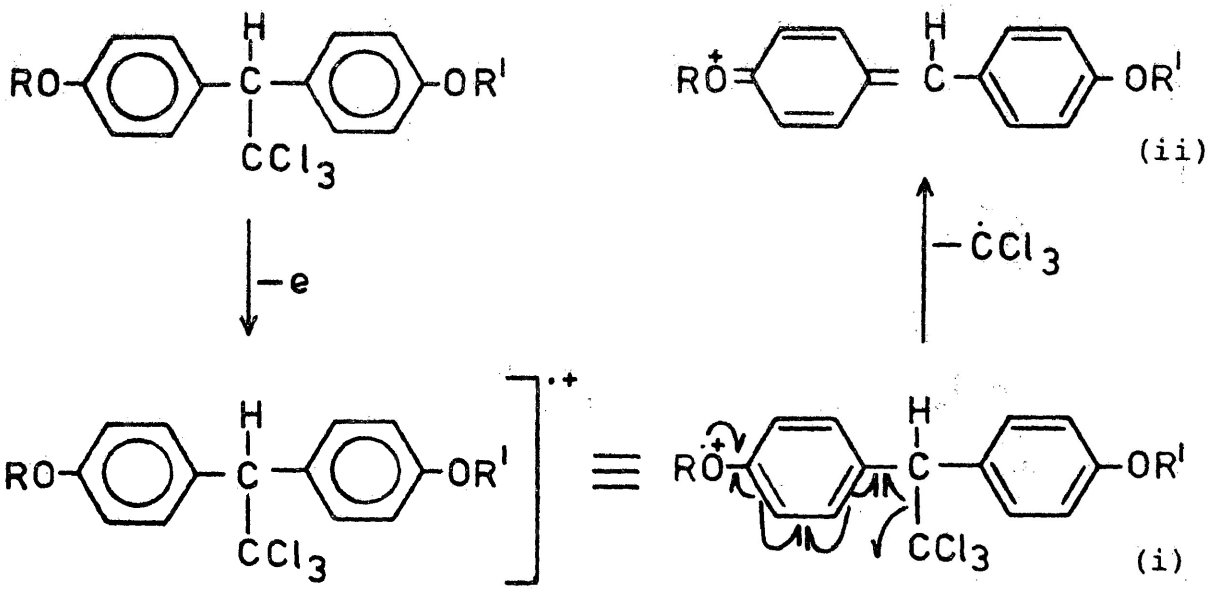


FIG. 8 Mass Spectrum of 4,4' dihydroxybenzophenone, (XIV).

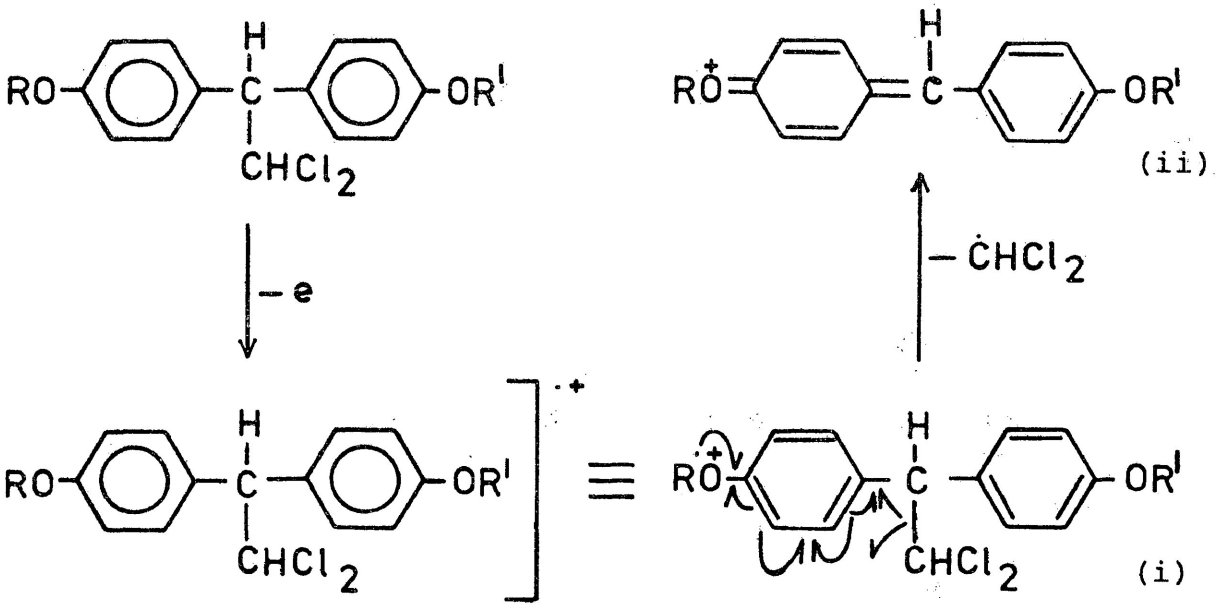
Scheme 5. Mass spectral fragmentation pattern proposed for the diaryltrichloroethanes I to IV.



(i) $R = R' = CH_3$: *m/e* 344; $R = CH_3$; $R' = H$: *m/e* 330;
$R = R' = H$: *m/e* 316; $R = R = C_2H_5$: *m/e* 372.

(ii) $R = R' = CH_3$: *m/e* 227; $R = CH_3$; $R' = H$: *m/e* 213;
$R = R' = H$: *m/e* 199; $R = R' = C_2H_5$: *m/e* 255.

Scheme 6. Mass spectral fragmentation pattern proposed for the diaryldichloroethanes VIII and IX.



(i) R = R' = CH_3: *m/e* 310; R = R' = H: *m/e* 282.

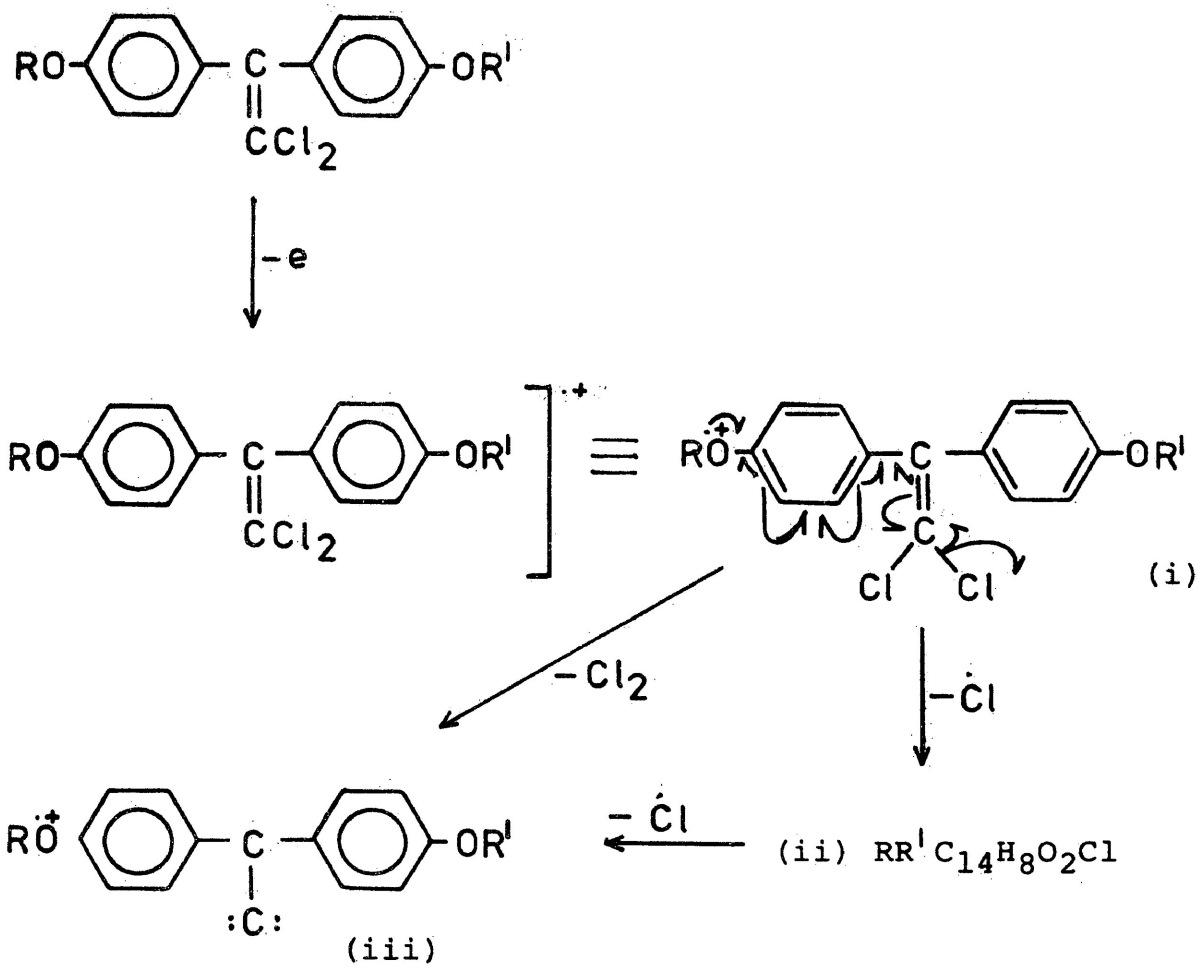
(ii) R = R' = CH_3: *m/e* 227; R = R' = H: *m/e* 199.

and 6. Similar schemes have been proposed for DDT and its dichloro derivative [DDD,(III) see p. 4](50). However, in the case of these compounds,fragments corresponding to the successive losses of the aryl chlorine atoms have also been postulated. There does not appear to be similar losses of the aryl moieties in the trichloro and dichloro compounds studied in the methoxychlor series.

In the case of two of the olefins (i.e. V and VII), the base peaks in the mass spectra are also the molecular ions. However in the spectrum of hydroxychlor olefin,(VI) the base peak may be seen as resulting from the loss of both chlorine atoms from the molecular ion: the base peak at *m/e* 210 clearly indicates the absence of chlorine atoms from this fragment [see p.62(iii) in Scheme 7]. The formation of this fragment which is also present in the spectra of the other two olefins, albeit in much lower intensity, could take place as indicated in Scheme 7. Whether or not the fragment (ii) is of major intensity or not may depend on the experimental conditions under which the spectrum is run, since MacNeil *et. al.* (11) found that (ii) is also the base peak in their spectrum of methoxychlor olefin,(V). This fragmentation pattern (Scheme 7) is analogous to that suggested for DDE,(II. see p. 4) (50).

In the spectrum of 4,4^{1}- dihydroxybenzophenone,(XIV)

Scheme 7. Mass spectral fragmentation pattern proposed for the diaryldichloroethylenes V to VII.

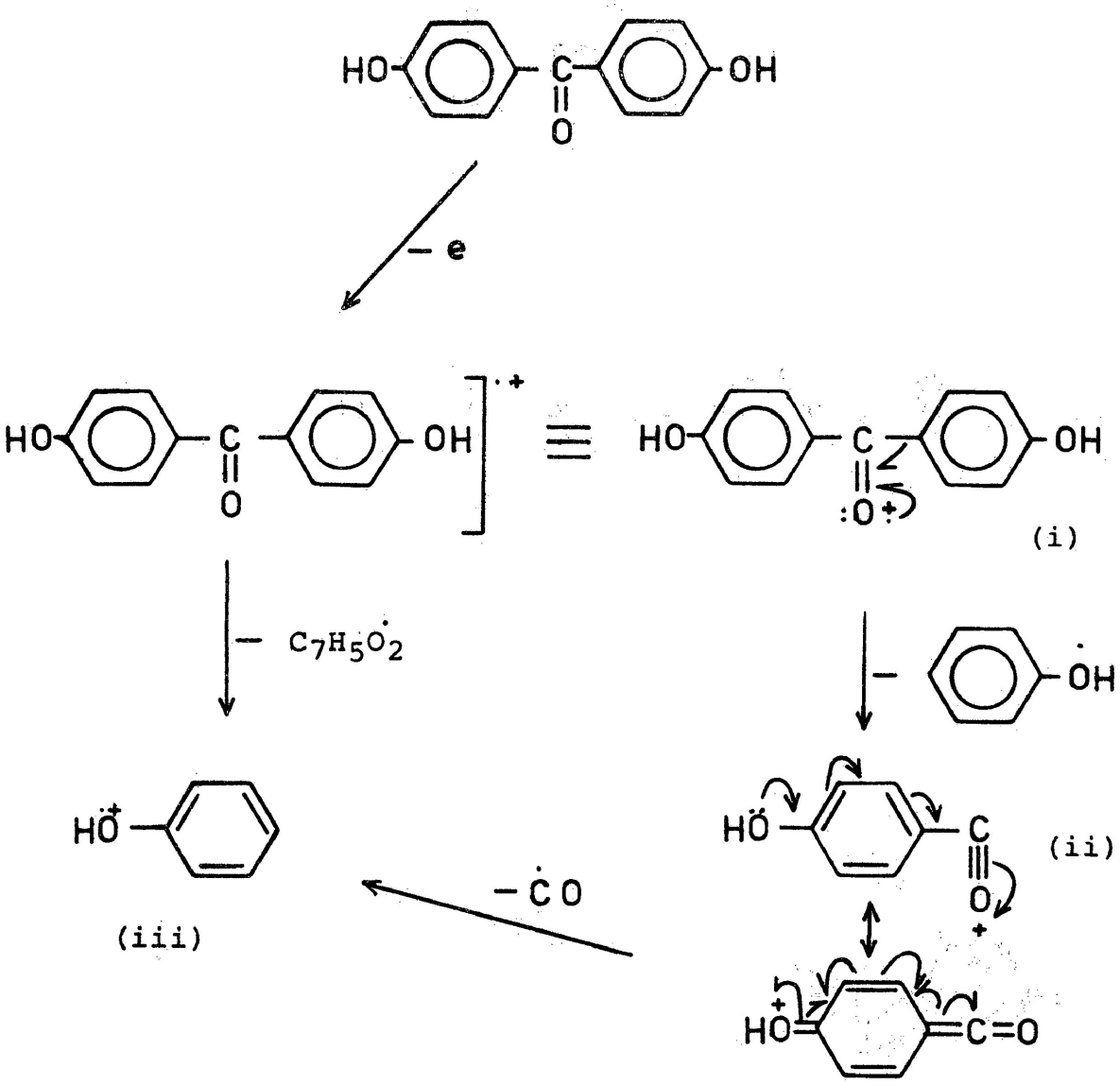


(i) R = R' = CH_3: *m/e* 308; R = CH_3; R' = H: *m/e* 294; R = R' = H; *m/e* 280.

(ii) R = R' = CH_3: *m/e* 273; R = CH_3; R' = H: *m/e* 259; R = R' = H: *m/e* 245.

(iii) R = R' = CH_3: *m/e* 238; R = CH_3; R' = H: *m/e* 224; R = R' = H: *m/e* 210.

Scheme 8. Mass spectral fragmentation pattern proposed for 4,4' - dihydroxybenzophenone, (XIV).



(i) *m/e* 214; (ii) *m/e* 121; (iii) *m/e* 93.

the fragment corresponding to the base peak may be formed as in Scheme 8 (i)→(ii), (i) being the molecular ion M^+ and (ii) the ion M^+-93. In addition minor fragments may be explained as in Scheme 8.

CHAPTER 3: THE BIODEGRADATION OF METHOXYCHLOR BY *KLEBSIELLA PNEUMONIAE*

Introduction. Studies concerning the microbial degradation of DDT and methoxychlor with bacteria are relatively scarce in the literature. Barker *et. al.* (51) have shown that the bacterium *Proteus vulgaris* is capable of converting *p,p'*-DDT to *p,p'*-DDD (see p.4 for structures). Wedemeyer (29,52) has also reported a similar conversion of DDT by the bacterium *Aerobacter aerogenes*, since renamed *Klebsiella pneumoniae*. Juengst and Alexander (30) have also investigated the conversion of DDT to water-soluble products by a number of bacteria isolated from sea water and marine sediments.

Investigations concerning the interaction of methoxychlor with bacteria include its degradation by a *Clostridium* species (18), its biomagnification by *A.aerogenes* and *Bacillus subtilis* (16) and its degradation in soils under flooded and submerged conditions, by Castro and Yoshida (17). Another study which included the interaction of methoxychlor with a bacterium was reported by Mendel *et. al.* (19). These authors investigated the interaction of *o,p'*-DDT and a number of DDT analogues, including methoxychlor, with *K.pneumoniae* (formerly *A.aerogenes*). They found that *o,p'*-DDT was converted to *o,p'*-DDD without exception, but as

discussed before (see p.10), they also reported that methoxychlor resists reductive dechlorination by this organism and that much of the methoxychlor was lost during the incubation period. It is known that both mammals (1) and fungi (see p.100 and 101) are capable of converting methoxychlor to hydroxychlor,(III) and methoxyhydroxychlor, (IV). Thus,the latter two compounds must also be considered as environmental pollutants. It was therefore considered quite possible that *K.pneumoniae* would be capable of converting methoxychlor, via the hydroxy derivatives III and IV, into a variety of products. Mendel *et. al.* (19) would not have recovered such polar metabolites (III and IV) with an extraction procedure using benzene. During the synthesis of these methoxychlor metabolites, it was found that hydroxychlor,(III) was virtually insoluble in benzene (see p.35). It was therefore decided to re-investigate the biodegradation of methoxychlor by this bacterium (*K.pneumoniae*) in an attempt to obtain more conclusive information on the environmental fate of this pesticide and the results of this reinvestigation are reported below.

Degradation of Methoxychlor by *K.pneumoniae*. Initial incubations of methoxychlor with *K.pneumoniae* cultures yielded extracts which, when analysed by both thin layer and gas chromatography, contained the dichloromethyl

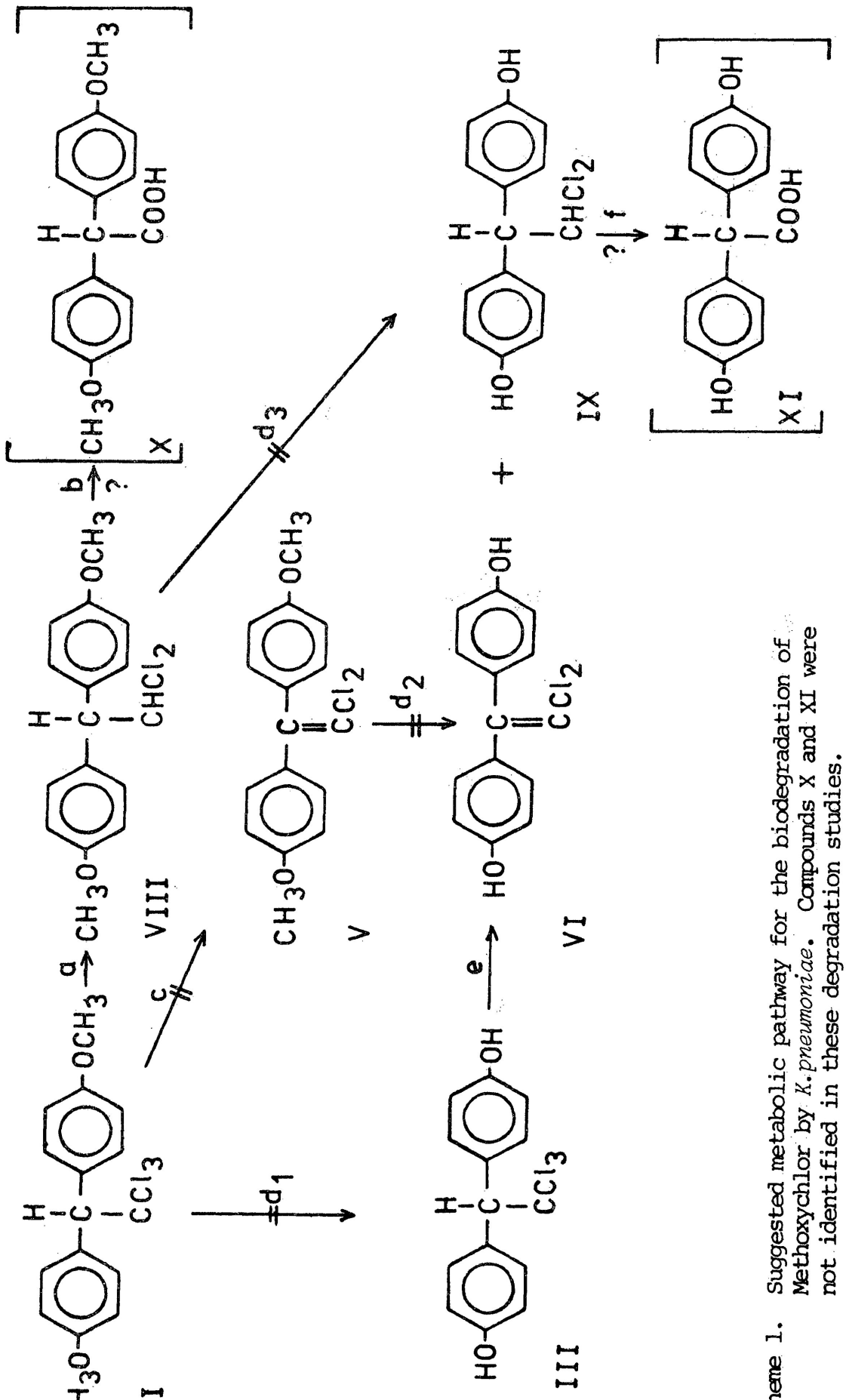
compound (VIII) as the only metabolite (step 'a' in Scheme 1*). The product of dehydrochlorination, i.e. methoxychlor olefin, (V) and the hydroxy compounds III, VI and IX were not found. Similar analysis of pesticide free cultures confirmed that no natural metabolites passed through a simple cleanup process designed for this system (see p.120). The application of this cleanup process to the extracts from incubations of methoxychlor with *K.pneumoniae* is illustrated in Fig. 9.

In subsequent incubations, the extracts of the cultures were analysed quantitatively for both unchanged starting material and for any metabolic alteration products found. The results of these analyses are summarized in Table 3.

It was found that in extracts of the cultures of *K.pneumoniae* incubated with methoxychlor (Exp. 1) that the metabolite found, i.e. VIII, accounted for only 15% of the administered pesticide of which 38% was recovered unchanged. Attempts were made to account for the "missing" methoxychlor by considering the following possibilities.

One possibility was to determine whether methoxychlor olefin, (V) was metabolised by this organism. If it is not degraded, it would be expected to accumulate in cultures of

* Schemes have been numbered per chapter (i.e., numbers for schemes in this chapter may not necessarily be the same as those in other chapters.).



Scheme 1. Suggested metabolic pathway for the biodegradation of Methoxychlor by *K.pneumoniae*. Compounds X and XI were not identified in these degradation studies.

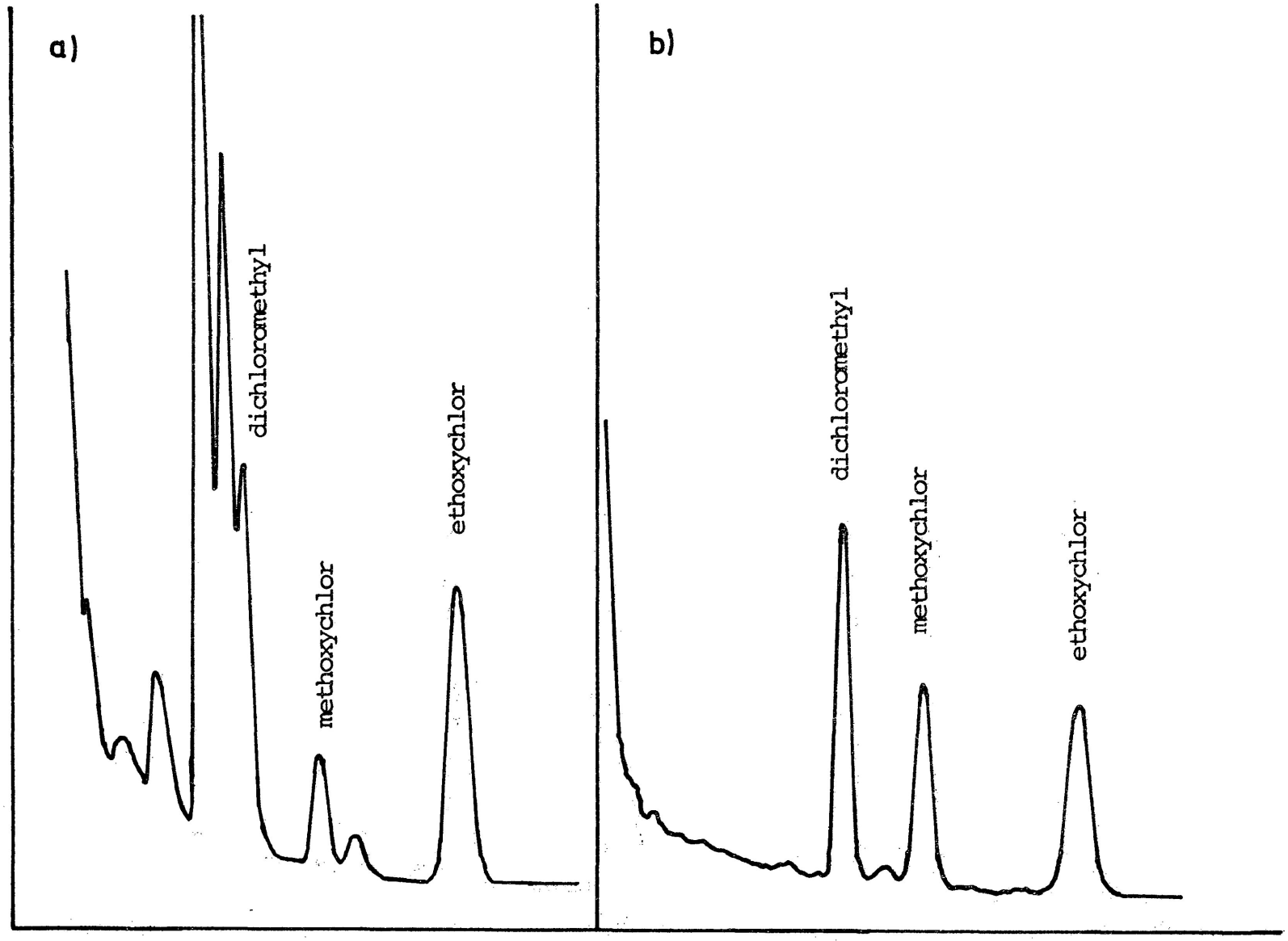


FIG.9 Result of cleanup process for extracts of *K.pneumoniae* incubated with Methoxychlor on the SE-30(5%) column. a) before cleanup; b) after cleanup.

Table 3. Quantitative analysis of extracts of *K.pneumoniae* incubated with methoxychlor and some of its derivatives.

Experiment No. [a]	Compound Added (mg.)	Addend Recovered [b] mg. (%)	Metabolite(s) Found (mg.)	Addend Equiv. Accounted for by Metabolite(s) mg.(%)
1a	Methoxychlor (2.0)	0.88 (44)	Methoxydichloro, (VIII) (0.28)	0.30 (15)
1b	Methoxychlor (2.0)	0.61 (31)	Methoxydichloro, (VIII) (0.26)	0.28 (14)
2a	Methoxychlor olefin, (V) (1.5)	1.42 (95)	-[c]	
2b	Methoxychlor olefin, (V) (1.5)	1.39 (85)	-[c]	
3a	Methoxydichloro, (VIII) (1.5)	1.06 (71)	-[c]	
3b	Methoxydichloro, (VIII) (1.5)	1.19 (80)	-[c]	
3c	Methoxydichloro, (VIII) (1.5)	1.12 (75)	-[c]	
4a	Hydroxychlor,(IV) (1.05)	0.18 (17)	Hydroxychlor olefin,(VI) [d]	-
4b	Hydroxychlor,(IV) (1.5)	0.22 (15)	and Hydroxydichloro,(IX)	-
5	Hydroxychlor olefin, (VI) (1.5)	1.5 (100)	-[c]	-
6	Hydroxychlor,(IV) (1.5)	0.24 (16)	Hydroxychlor olefin,(VI) (0.08)	0.08 (5)
			Hydroxydichloro,(IX) (0.23)	0.26 (17)
7	Hydroxydichloro,(IX) (1.5)	0.65 (43)	-[c]	-

[a] Average of triplicate analyses of *K.pneumoniae* extracts. [b] Average recovery efficiency of compounds (%): Methoxychlor, 75; Methoxychlor olefin,(V), 93; Methoxydichloromethyl,(VIII), 83 ; Hydroxychlor,(III), 72; Hydroxychlor olefin,(VI), 86; Hydroxydichloromethyl,(IX), 78%. [c] No metabolites found. [d] Qualitative identification only.

K.pneumoniae incubated with methoxychlor. The results from experiment 2 (Table 3) show that this olefin is not further degraded by this organism as it (the olefin) was recovered almost quantitatively from cultures of *K.pneumoniae* containing this compound. Thus, step 'c' in Scheme 1 does not appear to take place. It should be noted that the analogous product of DDT dehydrochlorination, the olefin DDE,(II,see p.4) resists further degradation by bacteria (29) and probably also in mammalian liver tissue (28).

Other possibilities were (i) that the dichloromethyl derivative,(VIII) formed in step 'a' (Scheme 1) is further degraded into unknown compounds (step 'b') such as the water-soluble diphenyl acetic acid,(X, in Scheme 1) or (ii) that ether cleavage (steps 'd_1' or 'd_3') could have led to the corresponding hydroxy compounds III and IX respectively. That (i) is the more probable follows from several observations.

The dichloromethyl compound,(VIII) was found to be further metabolised (Exp. 3) to an extent of 25-30%, but no degradation products were recovered (step 'b', Scheme 1).

When hydroxychlor,(III) was incubated with the bacterium (Exp. 4), between 80 and 90% was degraded, and qualitative examination of the extracts showed the presence of both hydroxychlor olefin,(VI) and the hydroxydichloromethyl derivative,(IX) as represented by step 'e' (Scheme 1).

This more efficient degradation of hydroxychlor,(III) means that this compound, if formed by ether cleavage (step 'd_1', Scheme 1), would not accumulate during incubation with methoxychlor as in Exp. 1. However, incubation of hydroxychlor olefin,(VI) with the bacterium (Exp. 5) shows that this compound, as the methoxychlor olefin,(V, Exp. 2) and DDE (29) is also resistant to further degradation. Thus, the complete absence of hydroxychlor olefin,(VI) from both experiments 1 and 2 leads to the conclusion that *K.pneumoniae* does not effect the cleavage of methoxy groups (steps 'd') in this series of compounds.

A quantitative assessment of the metabolism of hydroxychlor,(III) by the bacterium (Exp. 6), revealed that the resulting metabolites VI and IX account for only a small portion of the hydroxychlor,(III) that is degraded. As with the methoxydichloromethyl compound,(VIII) above, the hydroxydichloromethyl compound,(IX) could be metabolised to water-soluble metabolites such as the diphenyl acetic acid,(XI in Scheme 1). The results from Exp. 7 show that IX is degraded to an extent of 57% and no further degradation products were isolated. These results (Exp. 7) could therefore account for those obtained in Exp. 6. These last two experiments (6 and 7) also indicate that dehydrohalogenation of hydroxychlor,(III) to form the olefin, (VI) is a very minor reaction in the degradation scheme.

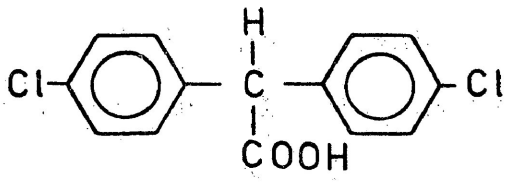
The above results demonstrate that *K.pneumoniae*, contrary to earlier reports (19), does effect the reductive dechlorination of compounds in the methoxychlor series. However, some final remarks are relevant at this time.

The water solubilities of methoxychlor and hydroxychlor,(III) have been reported as 0.1ppm (21) and 76ppm (1) respectively. Therefore, only a small percentage of the added methoxychlor is in solution at any given time, while the added hydroxychlor,(III) will be completely dissolved. This could be a probable reason for the more efficient degradation of this hydroxy compound, and no attempts were made to determine the true rates of these reaction by means of kinetic experiments.

Extracts resulting from experiments 4 and 6 (Table 3), i.e. metabolism of hydroxychlor,(III) contained an unknown compound not seen in the control cultures. This unknown compound interfered with gas chromatographic analyses, such that two different stationary phases were required. In these analyses the Dexsil 300 column could be used to analyse for hydroxychlor olefin,(VI) and hydroxychlor,(III) but not for the dichloromethyl compound,(IX) because the unknown compound had the same retention time as IX. The SE-30 column was used for the analysis of the dichloromethyl derivative,(IX) and hydroxychlor,(III) as on this column the retention time of the unknown was the same as that of

the hydroxychlor olefin,(VI). On thin layer chromatography (TLC) this unknown compound, was highly non-polar and did not give any of the typical colour reactions with the spray reagent used (1). It is unlikely then, that this compound is a metabolite of hydroxychlor,(III). One suggestion is that this compound is possibly a natural metabolite of the bacterium which is produced in response to the presence of the hydroxychlor,(III). The accumulation of this unknown from larger scale cultures will hopefully lead to its identification.

Finally, the degradation products of the dichloro compounds VIII and IX, which were not isolated with the procedures used, may well be the carboxylic acids X and XI (Scheme 1). 2,2-Bis (*p* - chlorophenyl) acetic acid,(XXII)



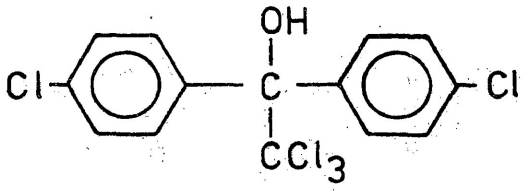
XXII 2,2-bis (*p* - chlorophenyl) acetic acid

the dichloro analogue of such acids has been isolated during investigations concerning the microbial degradation of DDT (28,30). Thus, further studies of *K.pneumoniae* interacting with methoxychlor and the derivatives III, VIII and IX

should be pursued in an attempt to isolate and identify such metabolites as the diphenyl acetic acids X and XI.

CHAPTER 4: INTERACTION OF METHOXYCHLOR WITH FUNGI

Introduction. Studies of the interaction of methoxychlor with fungi are virtually non-exsistent. However, there have been several studies done involving the interaction of DDT with a number of fungi. Kallman and Andrews (53), investigating the interaction of DDT with yeast, have found this organism capable of reductive dechlorination of this insecticide to its dichloromethyl derivative,(DDD,III, see p.4). Chacko and Lockwood (54) have found several actinomycetes to be effective in the degradation of DDT to DDD. These authors also reported that a strain of *Trichoderma viride* was ineffective in degrading this insecticide. Matsumura and Boush (35) have also studied the interaction of the soil fungus *T.viride* (Persoon) with DDT and found that it effectively degraded this compound to DDE,(II, see p.4) and to 1,1-bis (*p* - chlorophenyl)-2,2,2- trichloroethanol,(XXIII).

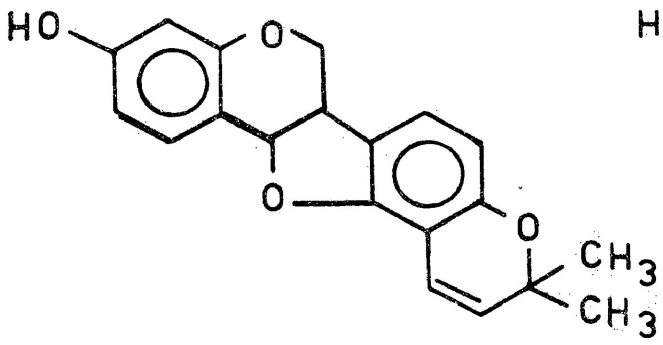


XXIII 1,1-Bis (*p* - chlorophenyl)-2,2,2- trichloroethanol

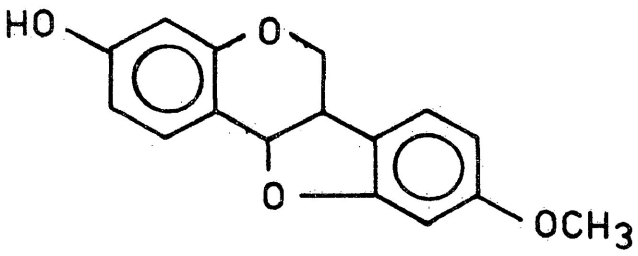
This α-hydroxylated DDT derivative was formed to a greater extent. Since it has been demonstrated that fungi can degrade DDT and because of the close relationship of this insecticide with methoxychlor, it is possible that similar biochemical transformations may take place when fungi interact with methoxychlor. Also, the methoxy groups of methoxychlor may provide an additional site of attack where fungi may be able to effect biochemical changes in this molecule. Thus, the investigation of such interactions with some common fungi is relevant for an assessment of the environmental impact of the pesticide methoxychlor.

Fungitoxicity Studies. Before such interactions are studied from a viewpoint of possible metabolic change in the methoxychlor molecule, it would be useful to obtain some information on the toxicity of various compounds in the methoxychlor series. Thus, the fungitoxicity of methoxychlor and its derivatives was studied, using the species *Mortierella isabellina*, *M.pusilla*, *Trichoderma viride*, and *Saprolegnia parasitica*, as described on p.20 and 21.

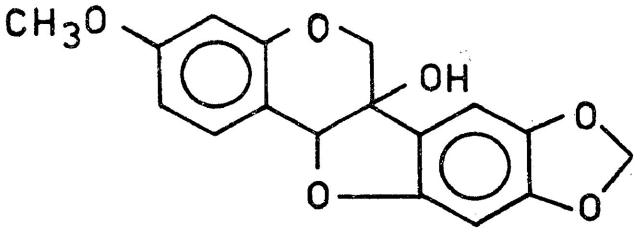
Fungitoxicity studies of various antifungal compounds have in recent years been concentrated, although not exclusively so, on the isoflavanoid phytoalexins (55,56,57). These are compounds produced in plant tissues possibly in response to microbial infection, and examples of such compounds are given (see p.78).



Phaseollin



Medicarpin

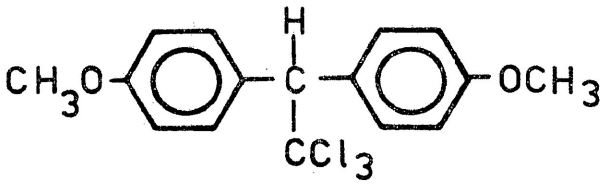


Pisatin

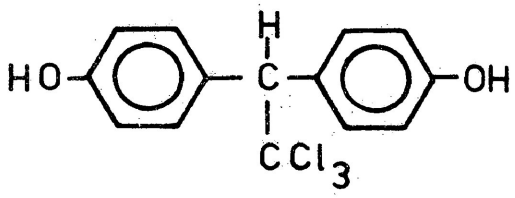
Although the reliability of fungitoxicity results, based on the technique of a single evaluation of mycelial growth, such as, colony diameter on agar plates, has been questioned (55), it was nevertheless decided to use this method, as described by Richardson and Miller (21). Bailey *et. al.* (55) have stated that such results are only valid when (i) there is no "lag-period" due to an initial

dying of mycelial cells within the inoculum, (ii) there is no depletion of the inhibitor in the medium resulting from metabolism at the growth front and (iii) that the rate of mycelial growth must remain constant throughout the period of the assay. However these objections to this technique do not necessarily hold for all fungitoxic compounds and that such an evaluation of inhibition of growth is acceptable in the present case was concluded from a number of initial observations.

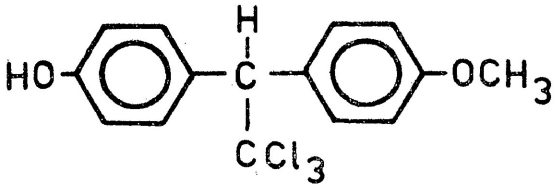
Measurements of colony diameter with time have shown that the growth of the test fungi in the presence of methoxychlor and its derivatives used in this investigation (see p.80) is linear, and no "lag-period" was observed. Examples of such growth curves are presented in Figs.10 and 11 . Also, preliminary liquid culture experiments (see p.97), involving extraction of possible metabolites, have shown that the metabolism of the test compounds is, at best very slow, and localized depletion zones were not observed on the agar plates. This indicates that the concentrations of the test compounds in the agar medium of a given plate remained essentially unchanged during the assay period. Finally, the plate culture bio-assay method allows measurements over a wide range (several orders of magnitude) of concentrations which is necessary from a viewpoint of environmental assessment.



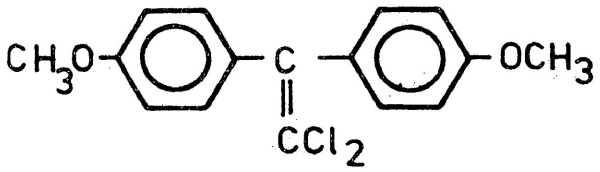
I



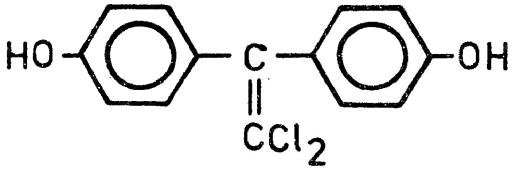
III



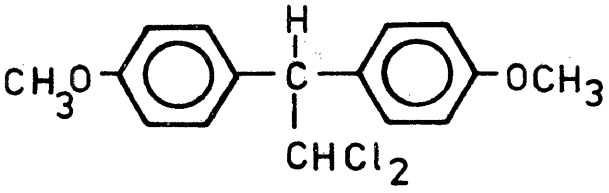
IV



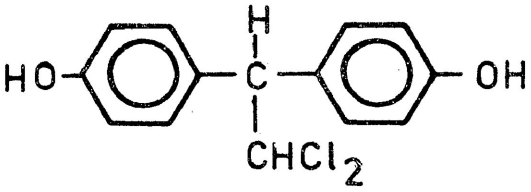
V



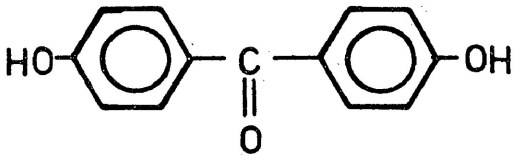
VI



VIII



IX



XIV

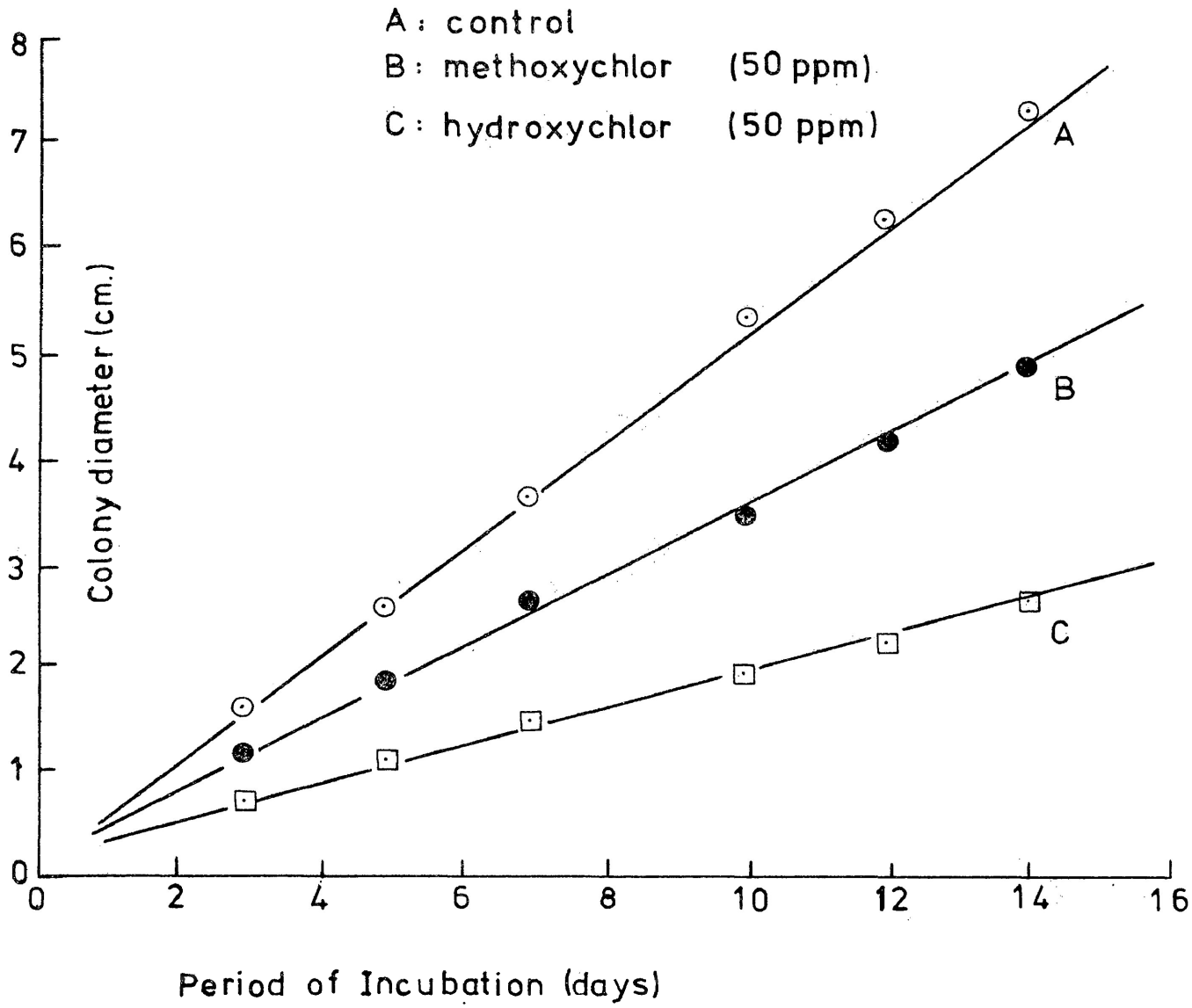


FIG.10 Growth (colony diameters on agar plates) of *Mortierella isabellina*: A - controls, B - 50ppm Methoxychlor,(I) and C - 50ppm of Hydroxychlor,(III).

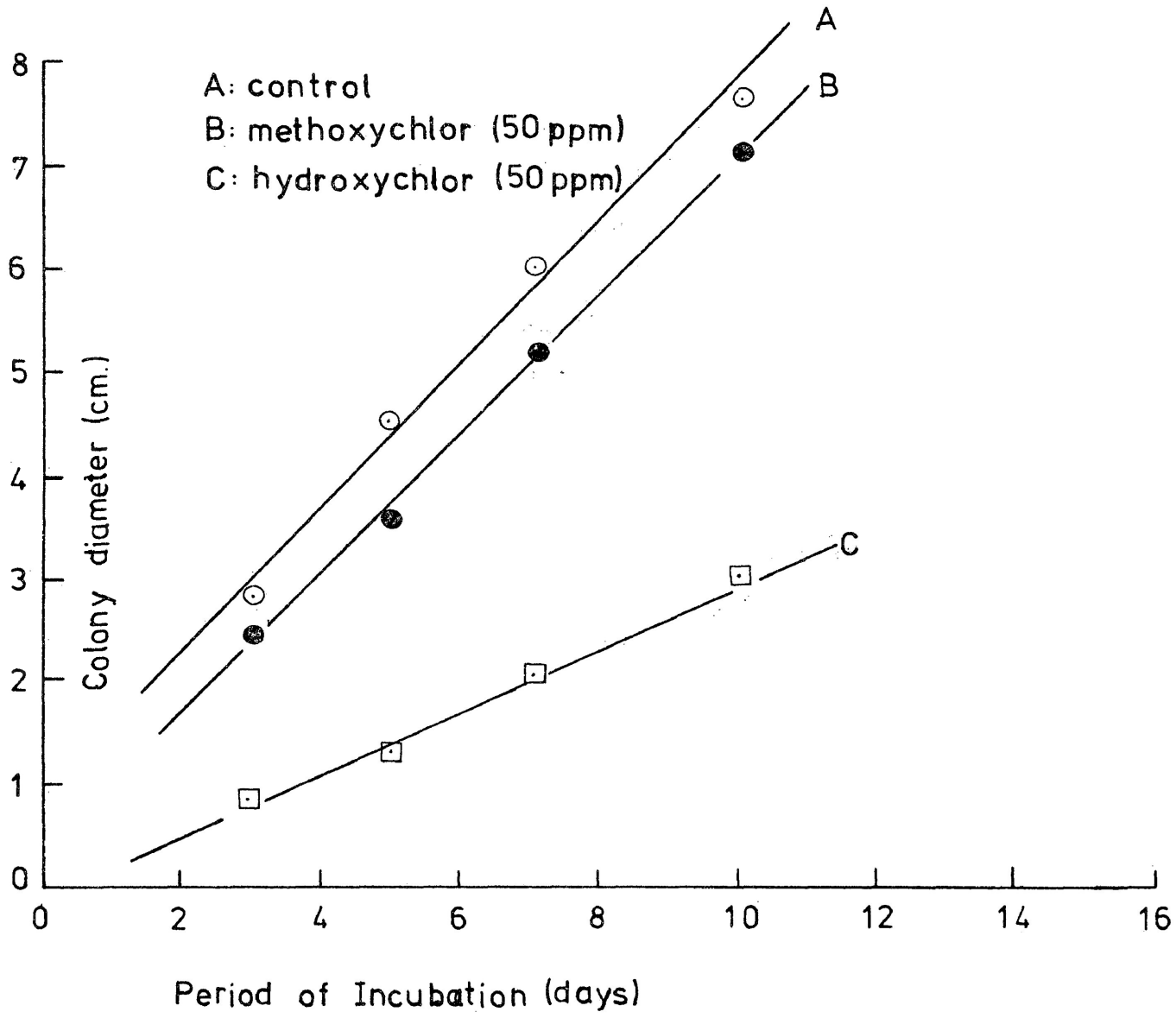


FIG.11 Growth (colony diameters on agar plates) of *Trichoderma viride*: A - controls, B - 50ppm of Methoxychlor,(I) and C - 50ppm of Hydroxychlor,(III).

The results (see below) have raised a matter of concern, in that there may be a need for fungitoxicity testing, not only of a given pesticide, but also of its metabolic and photochemical alteration products. This may lead to the production and subsequent publication of large numbers of dose-response relationships. Such dose-response relationships are traditionally presented as curves relating inhibition of mycelial growth with logarithmically increasing doses of pesticide in agar plates, such as illustrated in Figs.12 to 19. Therefore, a more concise format based on an analogy with methods of reporting spectroscopic data, is proposed here to report the data from these figures.

Ultraviolet spectroscopy may be used as an example for clarification (58). Thus, the ultraviolet spectrum of a compound may be given by stating the wavelength of maximum absorption λ_{max}, followed by the numerical value of the molar extinction coefficient ε.

The dosage-response curves as presented in Figs.12 to 19 can be similarly treated. The symbols used are D_{max} and i, where D_{max} is the dose in ppm at which maximum inhibition occurs, and i is the percentage inhibition observed at that dose. Using this notation, curve 1, Fig.12 (Methoxychlor versus *S.parasitica*) becomes $D_{max}3.1$; $i10$, which means that the dose causing maximum

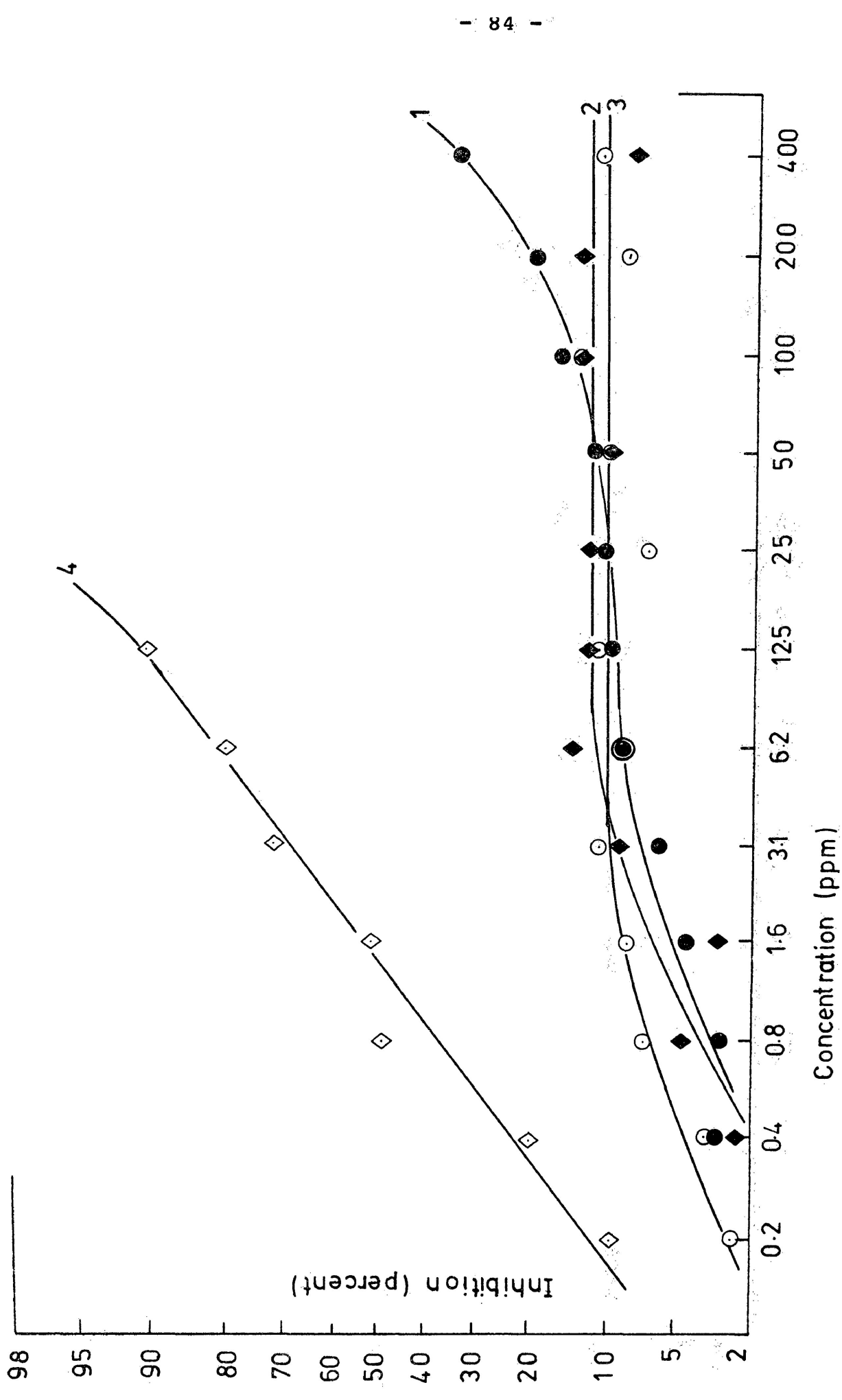


FIG. 12 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *Saprolegnia parasitica*
1: Methoxychlor, (I), 2: Methoxychlor olefin, (V), 3: Methoxydichloromethyl compound, (VIII), 4: Methoxyhydroxychlor, (IV).

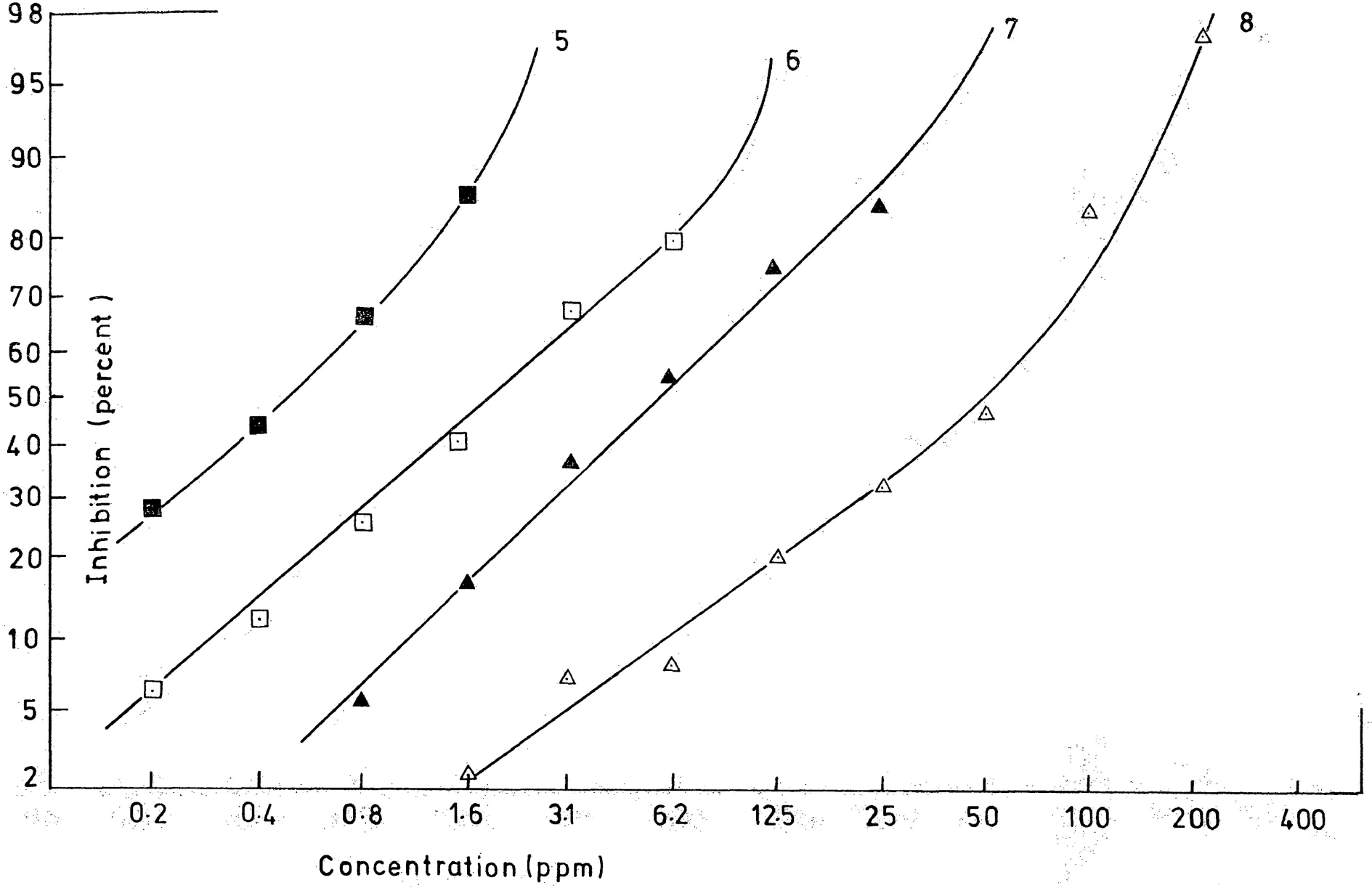


FIG.13 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *Saprolegnia parasitica*. 5: Hydroxychlor, (III), 6: Hydroxychlor olefin, (VI), 7: Hydroxydichloromethyl compound, (IX), 8: Benzophenone, (XIV).

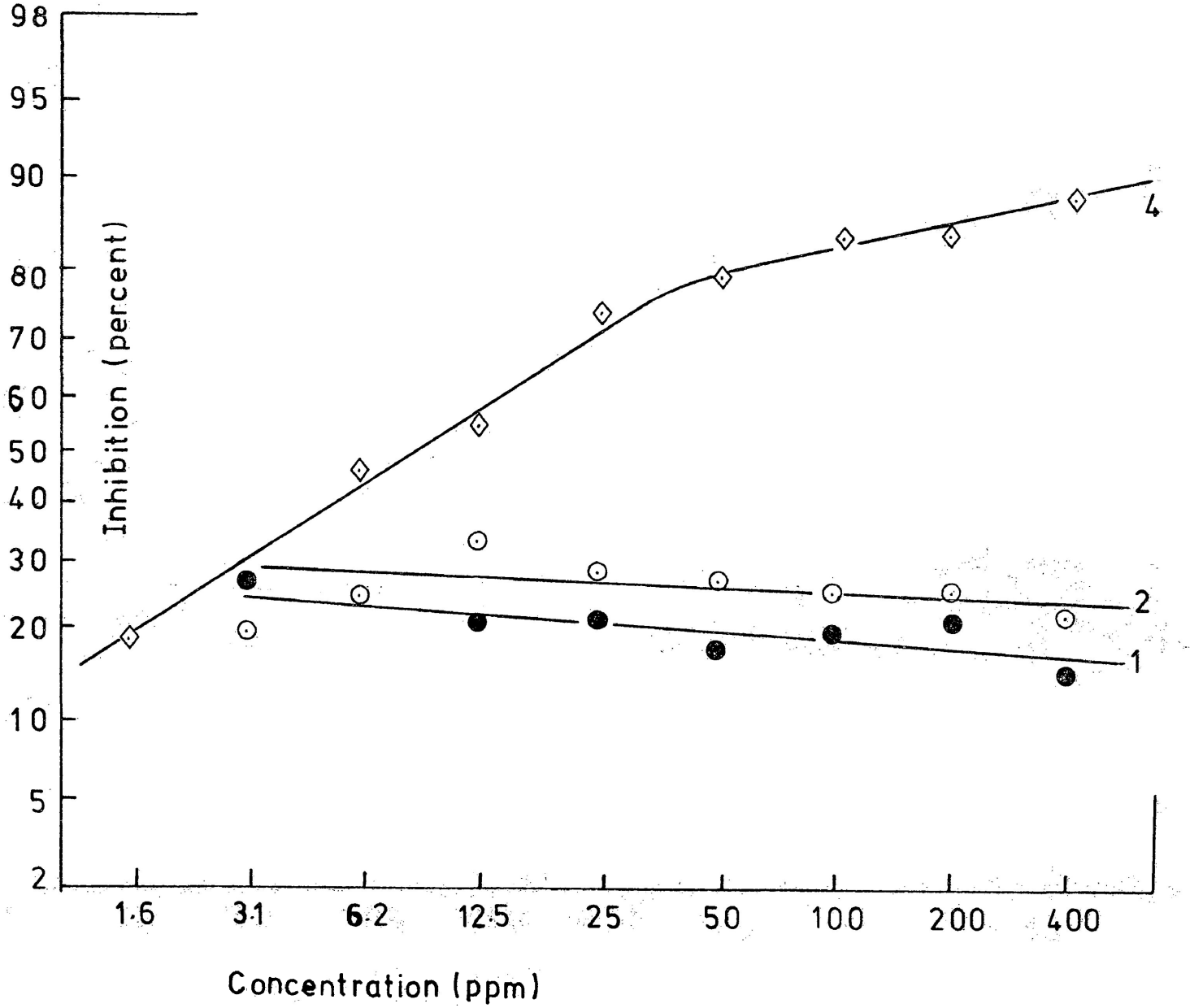


FIG.14 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *T.viride*. Numbering of curves as in Fig.12.

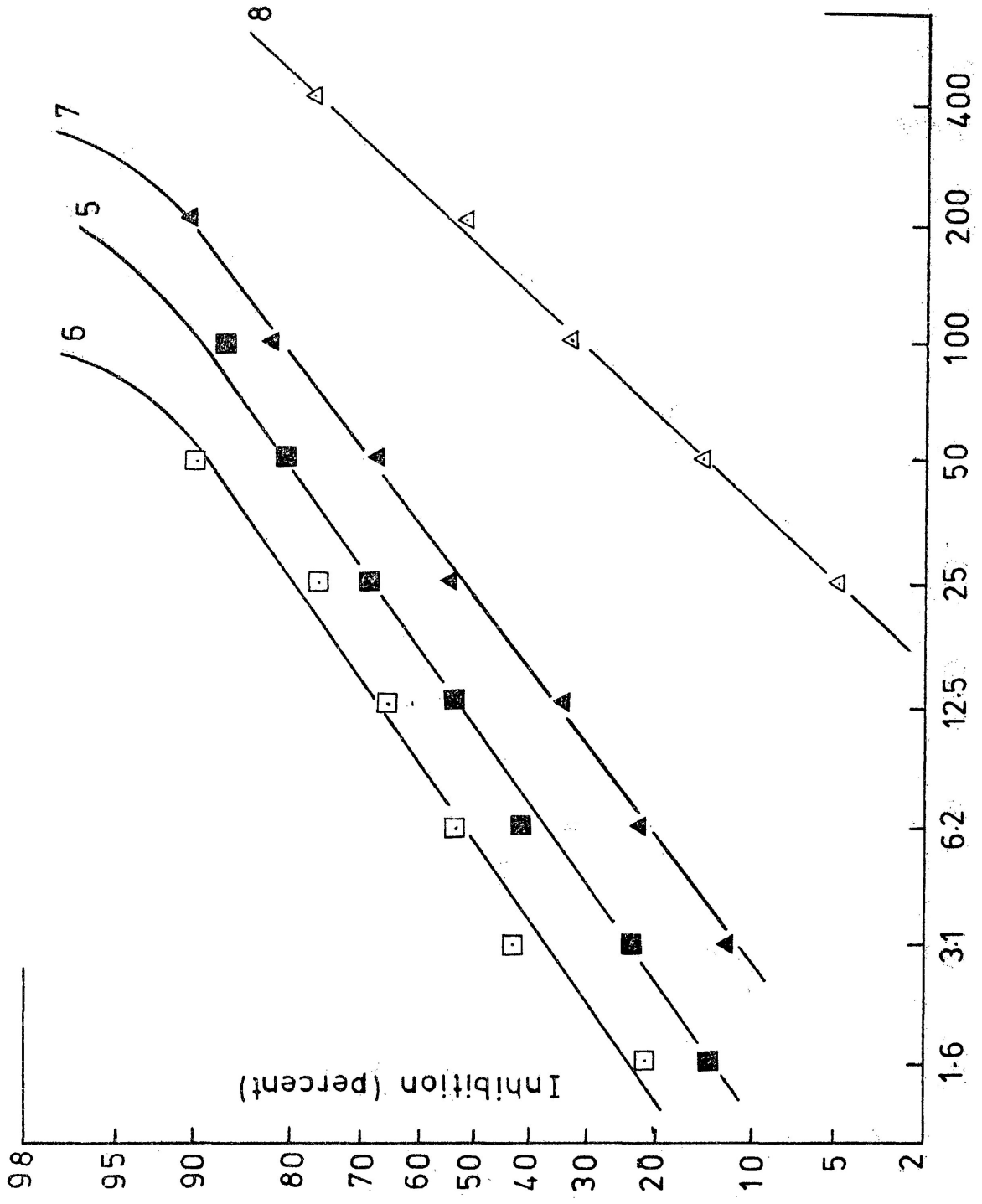


FIG.15 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *T.viride*. Numbering of curves as in Fig.13.

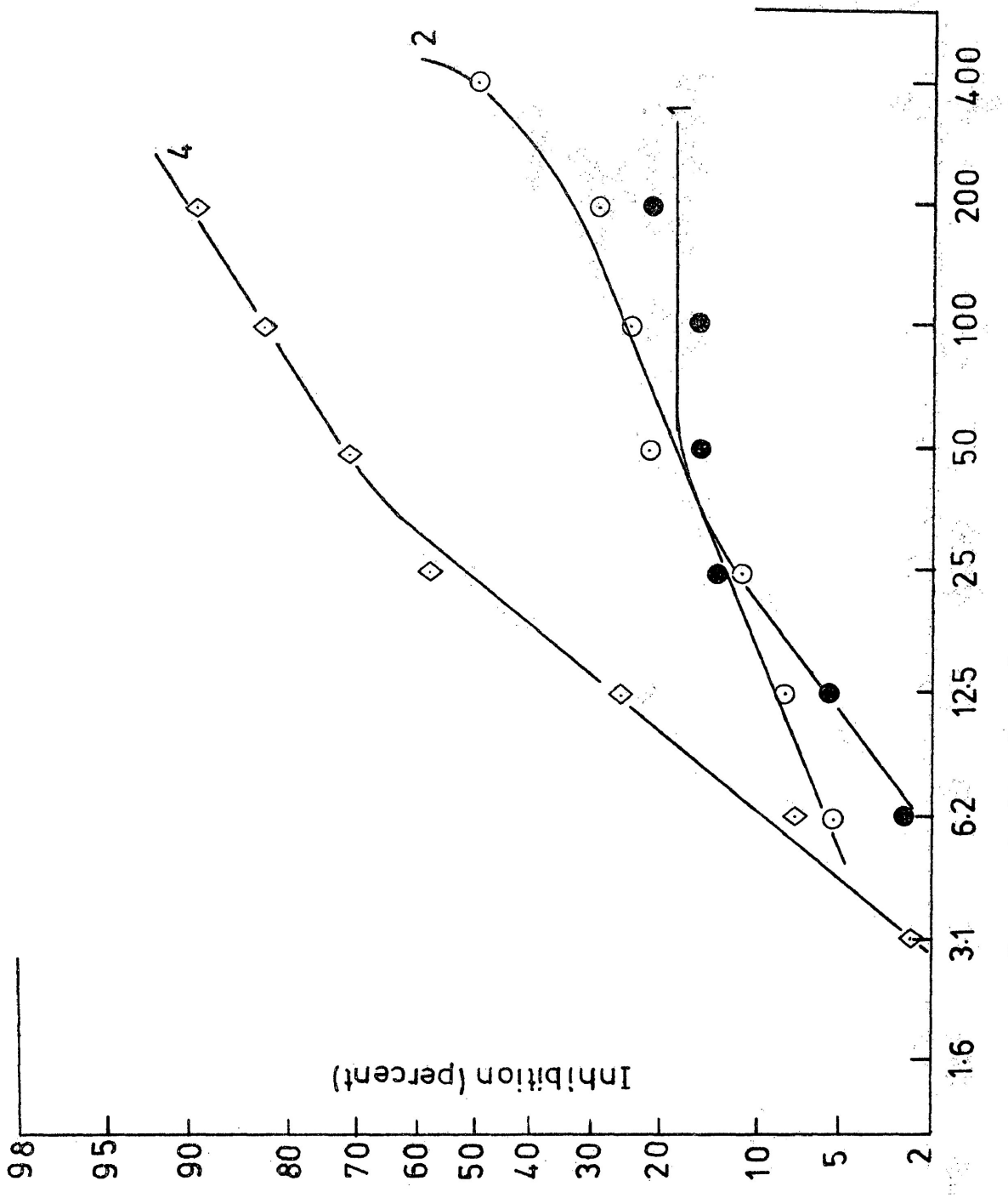


FIG.16 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *M. isabellina*. Numbering of curves as in Fig.12.

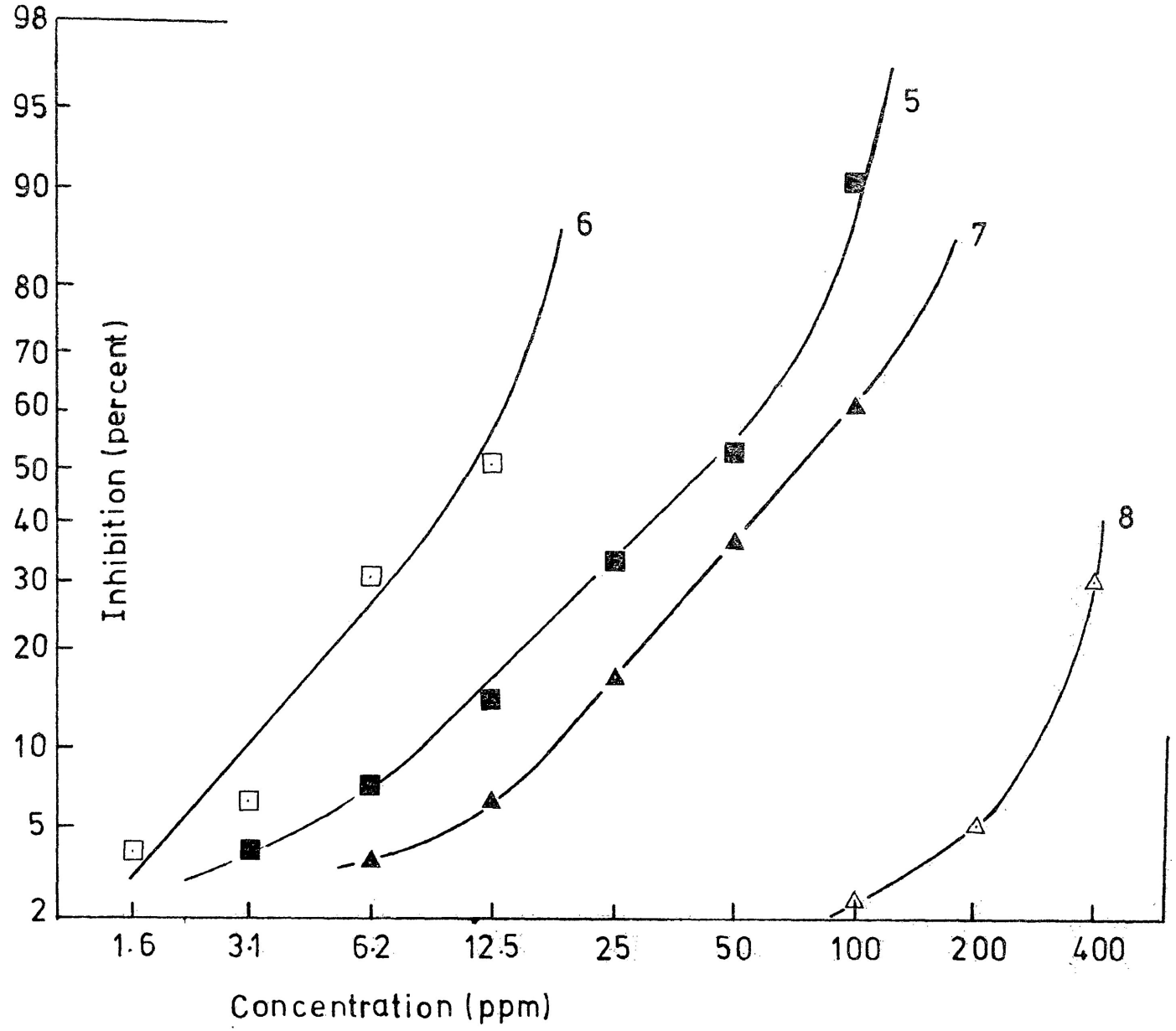


FIG.17 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *M.isabellina*. Numbering of curves as in Fig.13.

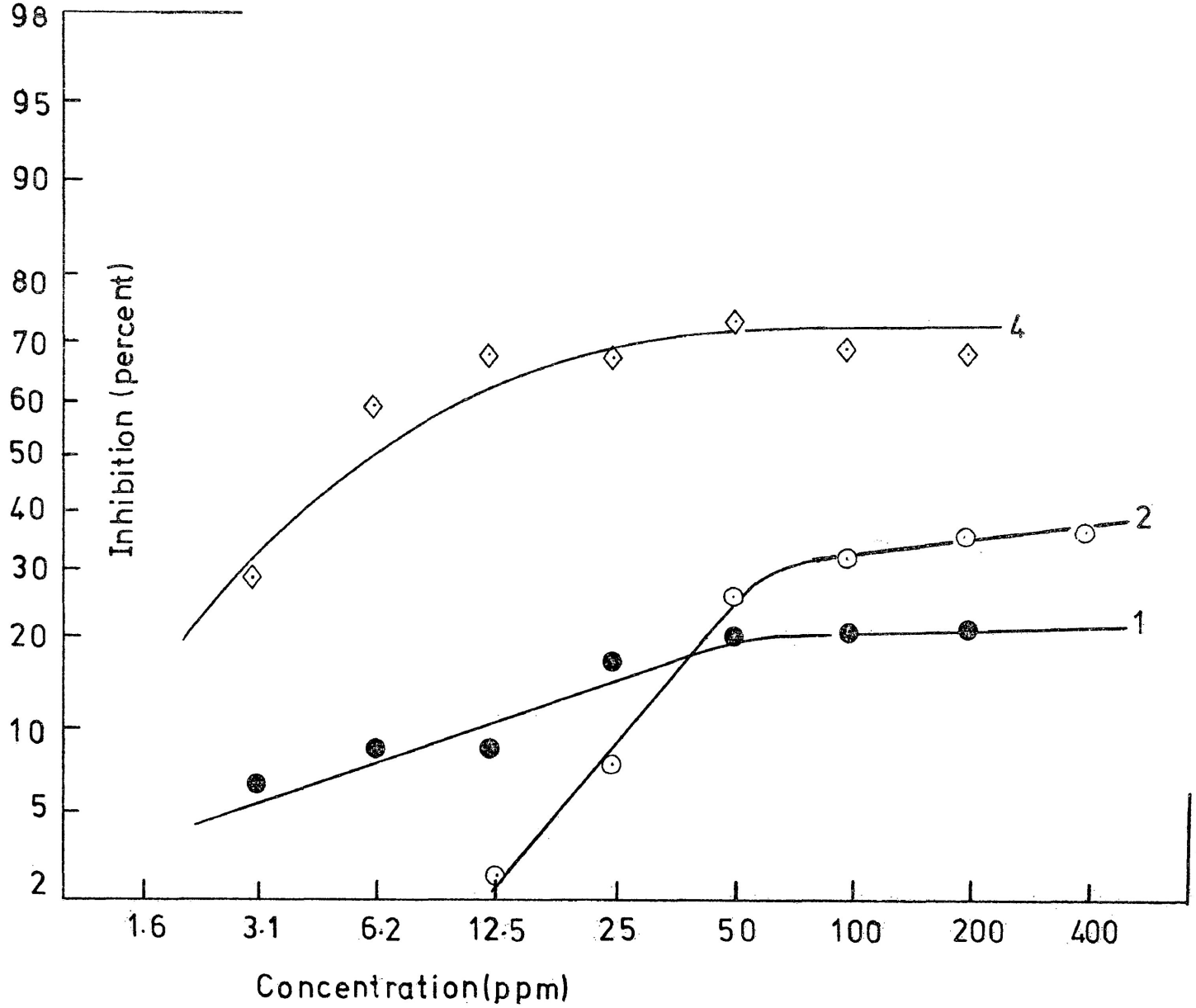


FIG.18 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *M.pusilla*. Numbering of curves as in Fig.12.

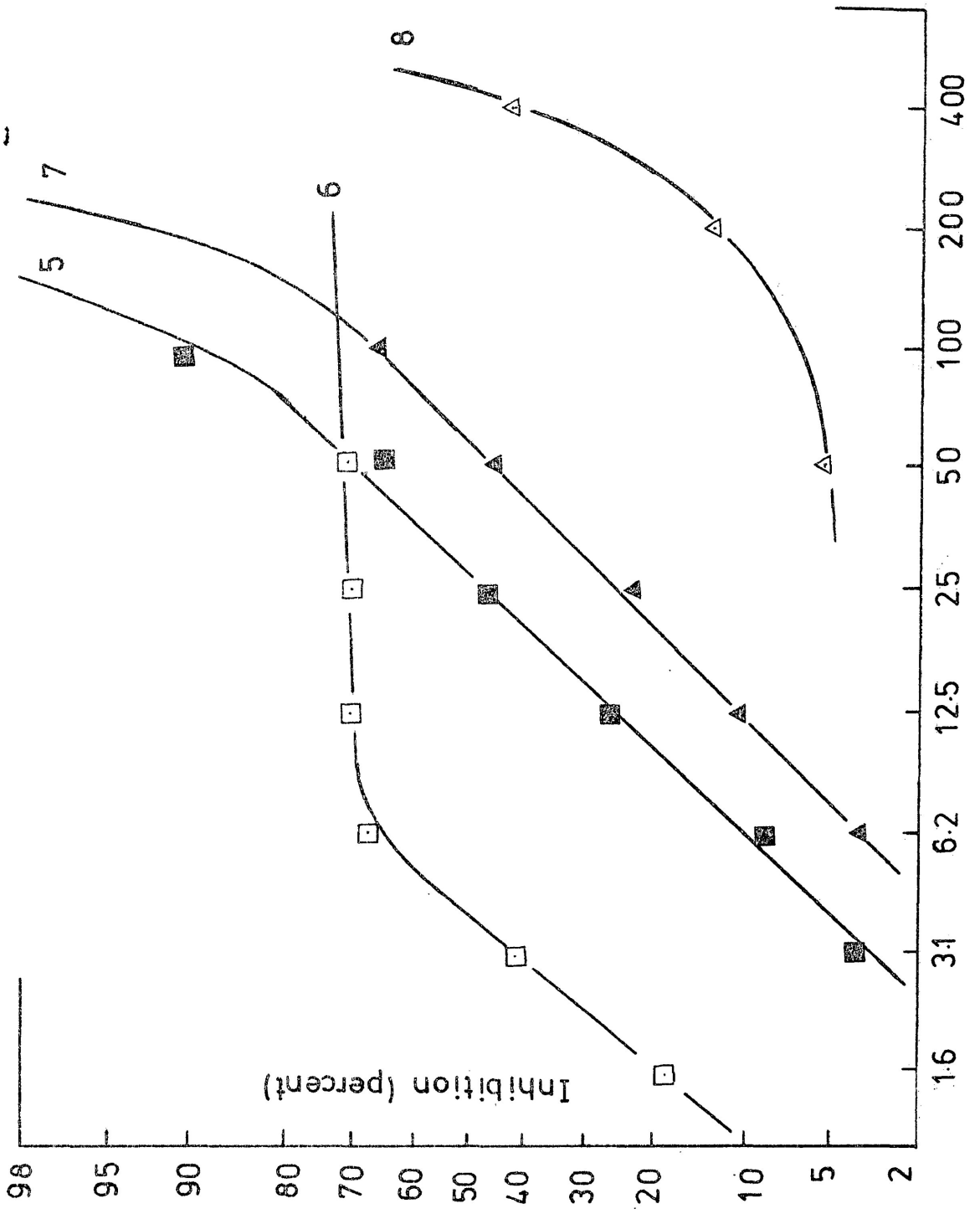


FIG.19 Dosage-response curves of Methoxychlor and some of its metabolites, assayed with *M.pusilla*. Numbering of curves as in Fig.13.

Table 4. Fungitoxicity of methoxychlor and some of its metabolites.

No.[a]	Compound		$D_{max};i$ [b]			
			S.p.[c]	*T.v.*[c]	*M.i.*[c]	*M.p.*[c]
I	Methoxychlor	(1)	400;35	[d];20	50;18	50;20
V	Methoxychlor olefin	(2)	3.1;10	[d];25	400;50	200;32
VIII	Methoxydichloromethyl	(3)	6.2;10	- [e]	- [e]	- [e]
IV	Methoxyhydroxychlor	(4)	25;100	400;88	200;88	12.5;73
III	Hydroxychlor	(5)	3.1;100	200;100	200;100	200;100
VI	Hydroxychlor olefin	(6)	12.5;100	100;100	25;100	12.5;70
IX	Hydroxydichlormethyl	(7)	50;100	400;100	200;100	200;100
XIV	Benzophenone	(8)	200;100	400;75	400;30	400;44

[a] Roman numerals refer to structural formulae on p.80; the arabic numbers in parentheses refer to the numbered curves in the respective figures (e.g. column one corresponds to Figs.12 and 13).

[b] The use of the notation $D_{max};i$ has been explained (see p.83).

[c] *S.p.* = *Saprolegnia parasitica*, *T.v.* = *Trichoderma viride*, *M.i.* = *Mortierella isabellina*, and *M.p.* = *M.pusilla*.

[d] These two dosage response curves consist approximately of horizontal lines at inhibition levels of 20 and 25% respectively throughout the range measured (i.e. 1.6-400ppm, see Fig.14).

[e] The points for these curves were erratic, and the curves were not drawn. The compound showed quite low inhibitory effects, ranging between (2-16)% for *T.viride*, less than 2% for *M.isabellina* and less than 5% for *M.pusilla* throughout the range measured (i.e. 1.6-400ppm).

inhibition is 3.1ppm and the *actual inhibition*, i, at that and higher doses is 10%.

From preliminary results of adsorption studies (W.H. Baarschers, unpublished results) and considering the low water solubilities of most of these compounds, toxicity measurements at concentrations greater than 400ppm do not appear meaningful. Therefore, dosage-response curves that do not reach a plateau in the range of concentrations used, are reported as $D_{max}400$, followed by the value of i at that concentration. One such case is curve 1, Fig.12 which is reported as $D_{max}400$; $i35$.

Finally, compounds which cause total inhibition of mycelial growth (i = 100%) at a given concentration (dose) in the range measured, are reported by giving the lowest D_{max} which causes such total inhibition. Thus, curve 4, Fig. 12 is reported as $D_{max}25$; $i100$.

In this way all the pertinent information in Figs.12 and 13 could be condensed into the first column in Table 4. Figs. 14 and 15 are likewise represented by the second column of this table and Figs. 16 to 19 are represented by the remaining two columns.

The results of this study presented in Table 4 differ from those given by Richardson and Miller (21), who studied the toxicity of several organochlorine pesticides (including methoxychlor) using the fungus *Rhizoctonia solani* (Kühn).

These authors found an anomalous feature in their dosage-response curves in that there is a peak at some point on the curve in every case. They explained the shape of these curves in terms of the water solubility of the pesticides, proposing that supersaturation occurs at some dose levels, followed by the observed crystallization of these compounds in plates containing higher dosages, leading to a lower actual concentration in the agar. It was found that for the fungi used in the present study, the methoxy compounds (I, V and VIII, see p.80) are less toxic than was found by Richardson and Miller (21) for *R.solani*. Also the hydroxy compounds (III, VI and IX) are considerably more water soluble than the methoxy compounds [Methoxychlor has water solubilities of 0.1ppm (21) or 0.62ppm (1) and the solubility of hydroxychlor is 76ppm (1)]. Thus, such anomalous dosage-response curves were not obtained for any of the compounds studied with respect to the fungi used.

The unsymmetrically substituted methoxychlor compound, (IV) had an inhibitory effect intermediary between the relatively low toxicity of the nonpolar dimethoxy derivatives (I, V and VIII) and the highly toxic, more polar dihydroxy derivatives (III, VI and IX), which is not surprising considering the relative polarities in this series.

The results reported above, show that the aquatic

species *S.parasitica* is clearly the most sensitive among the fungi used, particularly towards hydroxychlor,(III). This species would therefore be directly affected by water quality in terms of these pesticide derivatives and thus could conceivably be a good bio-indicator for the presence of compounds such as hydroxychlor,(III).

T.viride was found to be virtually unaffected by methoxychlor itself (curve 1, Fig.14). This indicates that this organism could possibly use this pesticide as a sole carbon source for its propagation. However, this aspect was not pursued further.

The culture plate bio-assay and its resultant dose-response relationship does not reveal the nature of the response in terms of its fungicidal or fungistatic nature. It was found that this question could be answered by a simple liquid culture technique.

Thus, liquid cultures containing appropriate concentrations of these compounds were incubated for periods of time so as to produce normal growth in a set of controls. Normal growth (in the absence of pesticide) was represented by the dry weight of the filtered mycelium (59). From flasks in which no growth was observed after this incubation period, inocula were removed, rinsed with sterile distilled water, and placed on pesticide free agar plates. When no growth was observed on these plates,

the compounds concerned were taken to be fungicidal to the fungus, and in those cases where the inoculum did grow on the plates, the compounds were taken to be fungistatic.

It was found that both compounds III and IX were fungicidal to *S.parasitica*. Both compounds were fungistatic to both *Mortierella* species at concentration levels of 50,100 and 200ppm, and *T.viride* experienced only fungistatic activity at 200ppm.

Only the two compounds III and IX were tested in this way, and it is probable that the other hydroxy derivative (VI), that caused total inhibition of mycelial growth at higher dosage levels, is also fungicidal to *S.parasitica* and fungistatic towards *T.viride* and the two *Mortierella* species. This compound (VI) was not further investigated because of its limited supply.

The results of this study show that the observations concerning so-called "soft" (i.e. biodegradable) pesticides, which are considered as good replacements for more environmentally persistent compounds, must be carefully monitored since *degradation products* of such replacements may have a greater potential to harm common soil and aquatic fungi in the environment than the parent compound. It is therefore suggested that in future investigations concerning pesticides and herbicides, more emphasis should be placed

on the environmental impact of the degradation products of such compounds.

As was noted earlier (see p.21) the above results have recently been published in a refereed journal (36).

Fungal Metabolism Studies. Since all fungi used in the fungitoxicity studies could tolerate methoxychlor itself to a reasonable extent, it is possible to study fungi in liquid culture in the presence of this pesticide with a view to isolate, or at least identify, possible metabolites. Preliminary studies of this kind have been conducted and are discussed below.

Initially, experiments designed for this purpose were of a qualitative nature. Thus, the two *Mortierella* species, *M.isabellina* and *M.pusilla* were grown in nutrient medium containing 10ppm of methoxychlor for periods of six, eight and ten days. The cultures were grown in duplicate for each incubation period, after which time the whole cultures were extracted with ethyl acetate. Thin layer chromatography (TLC) of all extracts showed the presence of residual methoxychlor and small amounts of methoxyhydroxychlor,(IV) and hydroxychlor,(III), and these identifications were confirmed by gas chromatography. Thus, these fungi do effect at least partial degradation of methoxychlor.

In a subsequent experiment, the *Mortierella* species

were grown in liquid culture containing an additional 50% of dextrose. As this increased quantity of dextrose caused increased mycelial growth (as determined from dry weight measurements), it was thought that there could be a corresponding increase in the metabolism of the methoxychlor, and therefore a greater concentration of methoxychlor was employed (20ppm). The cultures were grown for one week and extracted with ethyl acetate. TLC of the extracts from both fungi, followed by gas chromatography, confirmed the results of the first experiment. The increased amount of mycelium did not result in a visibly larger amount of metabolites formed.

The feeding of hydroxychlor,(III) to both *Mortierella* species did not lead to the observation of further metabolites. However, it cannot be stated with certainty whether this compound was metabolised at all, since no quantitative measurements were made of the amount of unchanged hydroxychlor,(III) remaining in the cultures.

A quantitative experiment was then attempted to determine the extent of methoxychlor degradation by these two fungi. *M.isabellina* was arbitrarily chosen for this experiment since the qualitative results were similar for both *Mortierella* species. Six flasks, containing 10ppm of methoxychlor in liquid cultures were incubated, three each for eight and twelve days respectively. Control cultures

were grown under the same conditions and 10ppm of the pesticide was added to these after the respective growth periods were completed. The mycelium from each culture was filtered and the aqueous portion extracted with ethyl acetate. The mycelium, in distilled water was blended for 1min. and extracted with ethyl acetate. The combined ethyl acetate extracts from each culture were analysed by gas chromatography. The recovery efficiency, as determined from the control cultures, varied widely and was unacceptably low (40-73% for the 8 day controls, 33-52% for the 12 day controls). Although it was evident that in the test cultures part of the methoxychlor was consumed, the results are inconclusive in view of those recovery efficiencies.

Several modifications in the experimental methods were tried in attempts to improve the recovery efficiency. However, these modifications, which included increased incubation periods and sonication of the mycelium, did not lead to conclusive results.

As the attempted quantitative experiments gave inconclusive results with respect to the amount of recovered methoxychlor, an experiment was designed to determine whether the loss was due to physical and/or mechanical means. It was found that methoxychlor could be recovered nearly quantitatively from the nutrient medium (which had not been inoculated), so that the low recovery efficiency is not due

to interaction with the nutrient chemicals or to the mechanics of the extraction procedure. Therefore it would appear that in addition to metabolic degradation of the pesticide, considerable amounts of methoxychlor are retained by the mycelium, probably as a result of adsorption. Such adsorption phenomena are known to take place with other micro-organisms, e.g. bacteria (16).

The quantitative aspect of the degradation of methoxychlor by the *Mortierella* species was not pursued at this time because of the above result. Therefore, it appears that in the degradation of methoxychlor by the *Mortierella* species, the methoxy substituents are the sites of attack for the enzyme systems of these fungi. Also the adsorption of the methoxychlor by the mycelium of these fungi means that such micro-organisms could present an environmental hazard to other organisms that feed on them.

Metabolic studies with methoxychlor and the aquatic fungus *S.parasitica* were confined to qualitative experiments, as similar adsorption problems were encountered. Thus, *S.parasitica* was grown in liquid cultures containing 10ppm of methoxychlor. After incubation, all cultures were worked up and prepared for analysis by TLC and gas chromatography. The results showed that all extracts of the fermentations, but not those of control cultures, contained

residual methoxychlor, methoxyhydroxychlor,(IV) and hydroxychlor,(III). Thus, this species can also effect at least partial degradation of the pesticide.

The results found from the metabolic investigations of *S.parasitica* with methoxychlor are similar to those for the *Mortierella* species, the only difference being in the relative amounts of hydroxychlor,(III) observed. The quantity of this metabolite appeared to be less in the case of *S.parasitica*.

Investigation of the metabolism of methoxychlor by the fungus *T.viride* was initially of a qualitative nature. Liquid cultures containing 20ppm of methoxychlor were incubated for a one week period, after which time the whole cultures were extracted with ethyl acetate. The extracts were analysed by TLC and all showed only a single blue spot with the spray reagent. The blue spot was more polar than methoxychlor itself, and the blue colour of this unknown compound suggested that it could be a dichloromethyl methoxychlor derivative. However, the R_f value did not correspond to the known compounds in this series (i.e. VIII and IX, see p.80).

The experiment was repeated with some modification, to determine whether intermediates to the above observed metabolite could be identified. Thus, six *T.viride* cultures containing methoxychlor were incubated for four, six and

eight days respectively. After extraction and TLC analysis, the extracts showed that after four days incubation only residual methoxychlor was present. But, after incubation periods of six and eight days, the unidentified compound (blue spot) and residual methoxychlor were observed. This confirmed the previous result, however, no other metabolites were observed.

When hydroxychlor,(III) was used instead of methoxychlor, to determine whether the observed blue spot was derived from this phenolic metabolite, it was found that only residual hydroxychlor,(III) was present in the extracts. It was concluded that hydroxychlor,(III) was not an intermediate in the formation of the observed unknown metabolite.

A series of quantitative experiments to determine the extent of methoxychlor metabolism by *T.viride* were attempted. The results were similar with respect to the amount of methoxychlor recovered. In all cases, a greater percentage of methoxychlor was recovered from cultures that were incubated in the presence of the pesticide than from control cultures to which the pesticide was added at the end of the incubation period. This indicates that the methoxychlor was retained by the mycelium of this fungus. However, the unidentified compound (blue spot) observed before, was not present in extracts of control cultures and is therefore probably a metabolite of methoxychlor produced by the fungus.

The unknown material corresponding to the blue TLC spot was then accumulated from larger scale cultures. After purification of the combined extracts by column chromatography and preparative thin layer chromatography, a white crystalline compound with m.p. 172-174° was obtained.

The mass spectrum, Fig.20 contained the molecular ion at M^+428(8%), and the base peak at *m/e* 249. From the molecular ion there were several consecutive losses of fragments of 18 mass units. This corresponds to the loss of water molecules and suggests that the unknown compound may contain an aldohexose group, possibly glucose, linked to an unidentified fragment with mass 249. It is quite possible that the unknown metabolite could be a glycoside as similar fragmentation patterns have been observed with a variety of naturally occurring glycosides (60). The unidentified fragment with mass 249 is formed by the loss of 179 mass units from the molecular ion. This loss of 179 mass units corresponds to the loss of the glucose part of such a glycoside.

The NMR of this unknown metabolite ($CDCl_3$) showed the presence of *p* - substituted aromatic rings together with signals that indicate the presence of a large number of aliphatic protons. The presence of the *p* - substituted aromatic rings may indicate that this compound is a

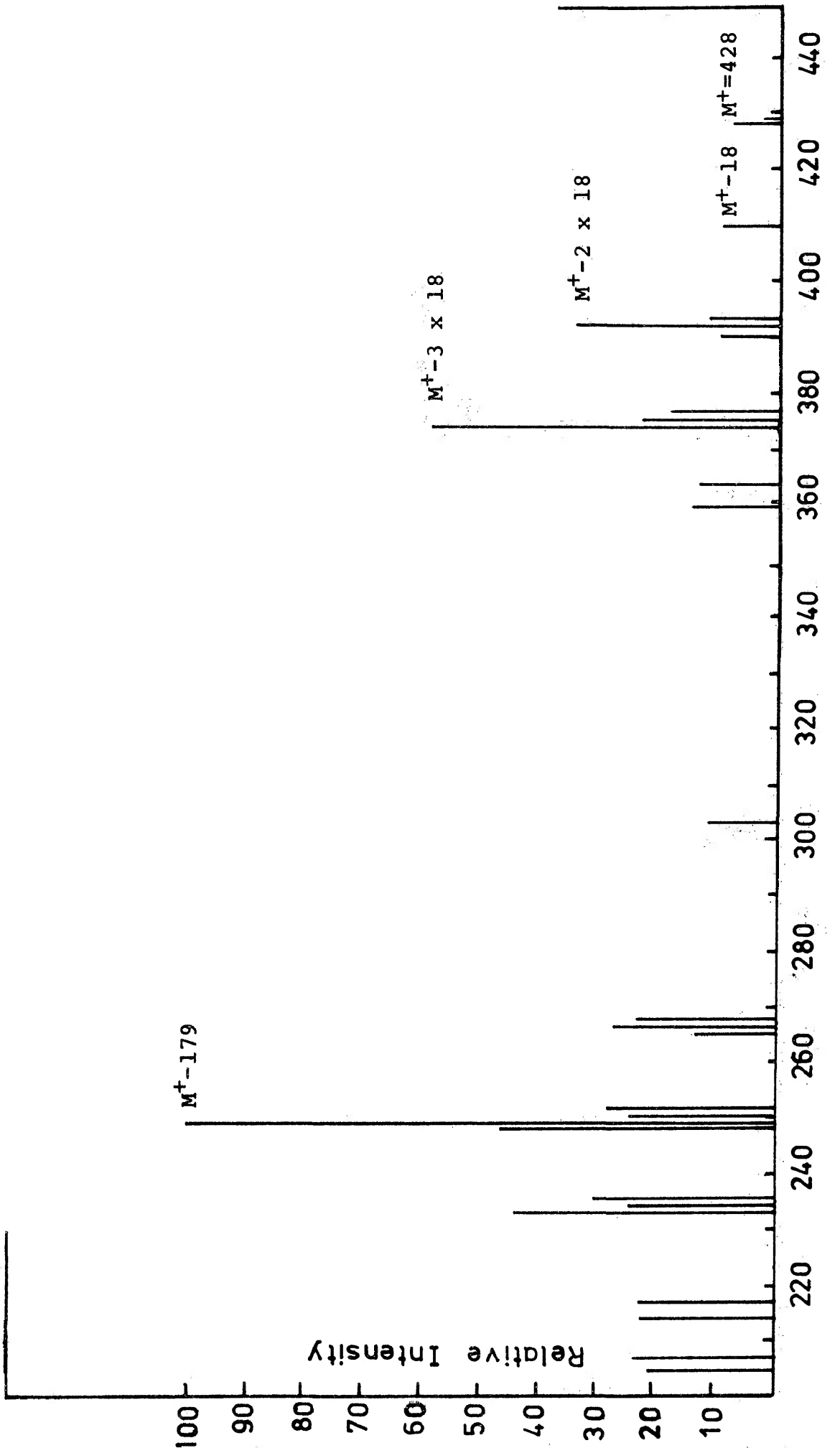


FIG. 20 Mass spectrum of unknown metabolite (blue spot) isolated from cultures of *T. viride*, incubated with methoxychlor.

metabolite of methoxychlor. Since the compound dissolves in chloroform, it is probably not a phenolic methoxychlor metabolite, as such compounds are virtually insoluble in this solvent. This is further substantiated from the degradation studies of *T.viride* with hydroxychlor,(III) since no other compounds other than the test compound were isolated from such cultures. In view of the above, it may be noted that the unknown compound could also be a natural metabolite produced by the fungus.

In conclusion, the interaction between *T.viride* and methoxychlor seems to produce at least one metabolite that can be isolated, though in very small amounts, indicating the very slow metabolism. Furthermore, there is evidence that the metabolite may possibly be a glycoside and that it is not a phenolic methoxychlor derivative. This species also tends to accumulate methoxychlor by adsorption and can present a similar environmental hazard as the two *Mortierella* species and *S.parasitica*.

Generally, the results obtained show that several fungi can metabolise methoxychlor, albeit very slowly, and like some other micro-organisms (16) they are capable of accumulating this pesticide by adsorption. This bio-concentration process could be an important avenue for the movement of this pesticide from one trophic level to the next, and this aspect of the environmental fate of

methoxychlor should be investigated further.

Finally, it is recommended that the location of the enzymes responsible for the ether cleavage in the methoxychlor molecule be investigated. For instance, such enzymes may be (i) bound to the cell walls, (ii) present in the cytoplasm or (iii) excreted into the culture medium at some stage during the growth of the fungi. The localization of these enzymes would facilitate further studies on these metabolic interactions.

CHAPTER 5: EXPERIMENTAL METHODS

(i) Analytical Methods

Nuclear magnetic resonance details of instrumentation; - nuclear magnetic resonance (NMR) spectra were measured with Varian A-60-A and Bruker Wp-80 spectrometers for deuterochloroform or *hexa*-deuteroacetone, as indicated, with tetramethylsilane as an internal reference at τ10.00ppm.

Mass spectral details of instrumentation; - mass spectra (MS) were recorded with an Hitachi-Perkin-Elmer RMU-7 double focusing mass spectrometer. Samples were introduced via the direct inlet at a normal ionizing voltage of 80ev.

Thin layer chromatography method; - the thin layer chromatography (TLC) technique employed was that described by Kapoor *et. al.* (1). Silicagel plates were eluted with three solvent systems: ether-pet. ether (30-60°) (1:9), ether-hexane (3:1) and pet. ether (30-60°)-chloroform-methanol (3:2:1). Spraying the plates with 0.5% diphenylamine and 0.5% zinc chloride (in acetone), heating at 110° for 10min., and exposing to ultraviolet radiation (254mμ) produced characteristic colours for these compounds. Grey-black colours were obtained for the diaryltrichloroethanes I to IV, pink colours for the diaryldichloroethylenes V to VII, and bright

blue colours for the diaryldichloroethanes VIII and IX, (see p.24 for structures I to IX).

Gas liquid chromatography (GLC) method; - a Perkin-Elmer model 3920B gas chromatograph equipped with dual flame ionization detectors (FID's) was used. Dual glass columns (6ft., ¼"i.d.) were used at temperatures from 220-250°, with nitrogen as a carrier gas at 25ml./min.. Liquid phases, on Gaschrom Q (80-100mesh) were Dexsil 300 (3%), Silicone gum SE-30(5%) and the mixed phases OV 17(3%)/ OV 101(3%) and SE-30(4%)/ OV 210 (6%). Qualitative identifications were achieved by determining retention times relative to DDT (see p.45, Table 1). Quantitative analyses were made by the use of internal standards [DDT or ethoxychlor,(II) p.24] [see also Takimoto *et. al.* (48)]. The internal standard stock solutions contained 125mg. of the relevant compound in 50ml. of ethyl acetate (i.e. 2.5mg./ml.). Calibration curves were prepared for the methoxy compounds (I, V and VIII, see p.24) by dissolving 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0mg. each in 1ml. of the internal standard solution, measuring the peak height ratios of the resulting chromatograms, and plotting the peak height ratios against the corresponding weight of the compound. Those compounds containing hydroxyl groups were first treated with Tri-sil (Pierce, 0.2ml.) and the resulting mixture made up to

1ml. by addition of 0.8ml. of the internal standard solution. New calibration curves were prepared for relevant compounds immediately prior to analysis of unknown samples obtained from the extraction of cultures.

(ii) Synthetic Chemistry

Melting points were determined with an electrothermal melting point apparatus and are corrected. Starting materials used in the various syntheses were commercially available and were used without further purification. All solvents were reagent grade and were used without further purification.

2,2-Bis (*p* - methoxyphenyl)-1,1,1- trichloroethane, (Methoxychlor,I), was supplied by Sigma Chemical Co. and was of two grades. Grade I (98% pure), m.p. 86-88° and grade II (90% pure), m.p. 81-83°, were both used during the present study without further purification. Gas chromatography showed that the impurities in these grades of methoxychlor were negligible for the grade I sample (only a single peak was observed) and was confined to a small single peak (approx. 5%) in the grade II sample. The NMR spectrum * ($CDCl_3$) of technical methoxychlor contained signals at τ6.26 (6H,2-OCH_3), τ5.03 (α-H) and

* A single asterisk denotes the NMR spectrum recorded on the Varian A-60-A spectrometer.

at τ2.4-3.4 (8H)ppm. The mass spectrum contained M^+ at *m/e* 344 (13%, $C_{16}H_{15}Cl_3O_2^+$), with the base peak at *m/e* 227 (100%, $C_{15}H_{15}O_2^+$).

2,2-Bis (*p* - ethoxyphenyl)-1,1,1- trichloroethane, (Ethoxychlor,II), was synthesized according to the method used for methoxychlor,(I) by Kapoor *et. al.* (1). Thus, phenetole (13.5g.) was mixed with chloral (8.0g., freshly distilled from a mixture of its hydrate and conc. sulphuric acid)in cold (0-5°), dry chloroform (300ml.) in nitrogen. Anhydrous aluminium trichloride (10.0g.) was slowly added with constant stirring, and the resulting mixture was further stirred for 1hr. in an ice bath. Stirring was continued for 16hr. at room temperature after which the reaction mixture was washed with water (300ml.) and the organic layer collected. The aqueous layer was extracted with chloroform (150ml.), the organic extracts were combined, dried (Na_2SO_4) and the solvent was removed. The crude product (11.2g.) was recrystallized from ethanol. The pure product had m.p. 105-106° [Lit.105°(38)]. The NMR spectrum ** ($CDCl_3$), contained a triplet at τ8.62 (6H), a quartet at τ6.0 (4H), a single peak at τ5.07 (α-H) and signals resulting from

** Double asterisks denote the NMR spectrum recorded on the Bruker Wp-80 spectrometer.

the aromatic protons at τ2.4-3.3 (8H)ppm. The mass spectrum contained M^+ at *m/e* 372 (2%, $C_{18}H_{19}Cl_3O_2^+$) with the base peak at *m/e* 255 (100%, $C_{17}H_{19}O_2^+$).

Analysis: Calcd. for $C_{18}H_{19}Cl_3O_2$: C,57.85; H,5.12; Found: C,57.59; H,5.21%.

2,2-Bis (*p* - hydroxyphenyl)-1,1,1- trichloroethane, (Hydroxychlor,III), was prepared by the same method as used above for ethoxychlor,(II). Phenol (13.7g.) was mixed with chloral (10.4g.) in cold (0-5°), dry chloroform (400ml.) in nitrogen. Anhydrous aluminium trichloride (10.6g.) was slowly added with constant stirring and the resulting mixture was stirred overnight (15hr.) at room temperature, then poured into water. The reaction mixture was worked up as above and the combined crude product (5.98g.) was recrystallized from a benzene-ethanol mixture to give hydroxychlor as white crystals, m.p. 208° [Lit.194°(1),212°(38)]. The NMR spectrum ** (C_2D_6CO) contained signals at τ7.1 (2H, exchangeable with D_2O), τ4.85 (α-H) and at τ2.3-3.2 (8H)ppm. The mass spectrum contained M^+ at *m/e* 316 (10%, $C_{14}H_{11}Cl_3O_2^+$) with the base peak at *m/e* 199 (100%, $C_{13}H_{11}O_2^+$).

Analysis: Calcd. for $C_{14}H_{11}Cl_3O_2$: C,52.95; H,3.49; Found: C,52.79; H,3.48%.

2-*p*-methoxyphenyl-2-*p*-hydroxyphenyl-1,1,1-trichloroethane, (Methoxyhydroxychlor,IV) was prepared by condensing *p* - methoxyphenyl trichloromethyl carbinol, (XV, see p.25) with phenol. *p* - Anisaldehyde (18.4g.) was added to dry chloroform (25ml.), and to this solution was added potassium hydroxide (1.6g.) during a 1hr. period with constant stirring. Stirring was continued for 3hr. at room temperature. Anhydrous ether (80ml.) was added and the mixture filtered. The excess ether and chloroform were removed from the filtrate and the residue was steam distilled to remove excess aldehyde and any *p* - chlorobenzoic acid that might have been produced. The non-volatile residue of the steam distillation was extracted with ether (3 x 100ml.) and the ether extracts were washed with aqueous sodium hydroxide (2%). The ether extracts were then dried (Na_2SO_4) and the ether was removed. The resulting crude product was distilled under reduced pressure to give the carbinol,(XV), (4.8g.), b.p.140-145° (1.5mm.). The NMR spectrum * ($CDCl_3$) contained signals at τ6.66 (1H,-OH), τ6.22 (3H,-OCH_3), τ4.86 (α-H) and at τ2.4-3.2 (4H). The carbinol (XV,2.0g.) was added to phenol (4.3g.) in cold (0-5°) dry chloroform (25ml.) in nitrogen. Anhydrous aluminium trichloride (0.5g.) was added with constant stirring and the resulting mixture was stirred

for 16hr. at room temperature. The deep purple solution formed was washed with water and the organic layer was collected, the solvent removed and excess phenol was distilled off *in vacuo*. The non-volatile residue was extracted with ether (2 x 100ml.), the combined ether extracts were dried (Na_2SO_4) and the solvent was removed, giving a viscous reddish brown oil (2.9g.). Thin layer chromatography (TLC) indicated the presence of a number of unknown byproducts from which the product could be isolated by column chromatography on Silicagel (90g., column i.d., 2.5cm.). Elution with a mixture of pet. ether (30-60°) and chloroform, beginning with a 3:2 ratio and increasing the concentration of chloroform to one hundred percent, gave the product (1.6g.) which after recrystallization from a chloroform-cyclohexane mixture (2:1) had m.p. 118-119° [Lit.112-114°(1),113-115°(37)]. The NMR spectrum ** (C_2D_6CO) contained signals at τ7.05 (1H,-OH), τ6.2 (3H,-OCH_3), τ4.85 (α-H) and at τ2.3-3.2 (8H). The mass spectrum contained M^+ at *m/e* 330 (28%, $C_{15}H_{13}Cl_3O_2^+$) with the base peak at *m/e* 213 (100%, $C_{14}H_{12}O_2^+$).

Analysis: Calcd. for $C_{15}H_{13}Cl_3O_2$: C,54.33; H,3.95; Found: C,54.28; H,4.04%.

2,2-Bis (*p* - methoxyphenyl)-1,1- dichloroethylene, (Methoxychlor olefin,V), was synthesized according to

the method of Kapoor *et. al.* (1). Technical grade methoxychlor (3.45g.) was refluxed in ethanol (100ml.), containing potassium hydroxide (0.86g.) for 1.5hr.. The cooled reaction mixture was poured into water and the resulting mixture was extracted with chloroform (100ml.). The organic extract was dried (Na_2SO_4) and the solvent removed, giving the product (1.97g.) which after recrystallization from ethanol had m.p. 112-114° [Lit.109°(37),114-114.5°(19)]. The NMR spectrum* ($CDCl_3$) contained signals at τ6.25 (6H,2-OCH_3) and at τ2.7 -3.25 (8H). The mass spectrum contained M^+ at *m/e* 308 (100%, $C_{16}H_{14}Cl_2O_2^+$) with a major fragment at *m/e* 238 (42%, $C_{16}H_{14}O_2^+$).

Analysis: Calcd. for $C_{16}H_{14}Cl_2O_2$: C,62.14; H,4.57; Found: C,62.46; H,4.85%.

2,2-Bis (*p* - hydroxyphenyl)-1,1- dichloroethylene, (Hydroxychlor olefin,VI), was synthesized by two methods.

Method I (1). A mixture of hydroxychlor,(III, 3.2g.), aqueous ethanol (100ml., 50%) and potassium hydroxide (2.2g.) was refluxed for 4hr.. Water (25ml.) was added, the resulting mixture was acidified to pH2.0 and extracted with ethyl acetate (2 x 100ml.). The combined organic extracts were dried (Na_2SO_4) and the solvent removed. The residue, a reddish brown oil, was subjected to column chromatography (85g. Silicagel;

column i.d., 2.5cm.). Elution with a chloroform-benzene mixture (from 3:1 to 100% chloroform) gave the desired product (0.74g.) which after recrystallization from aqueous ethanol (20%), yielded white crystals m.p. 215° [Lit.212°(1)]. The NMR spectrum ** (C_2D_6CO) had signals at τ7.02 (2H,-OH) and at τ2.8-3.3 (8H). The mass spectrum contained M^+ at *m/e* 280 (77%, $C_{14}H_{10}Cl_2O_2^+$) with the base peak at *m/e* 210 (100%, $C_{14}H_{10}O_2^+$).

Analysis: Calcd. for $C_{14}H_{10}Cl_2O_2$: C,59.81; H,3.59; Found: C,59.15; H,3.69%.

Method II (45). Hydroxychlor,(III,1.0g.) was refluxed in 3N methanolic potassium hydroxide (50ml.) for 0.5hr.. The reaction mixture was added to cold water (150ml.) and acidified. The precipitated product was isolated by filtration (0.51g.) and after recrystallization from aqueous ethanol (20%), had m.p. 215°. NMR, mass spectral and analysis data were identical to those obtained above.

2-*p*-methoxyphenyl-2-*p*-hydroxyphenyl-1,1- dichloroethylene, (Methoxyhydroxychlor olefin,VII), was synthesized according to the method of Hubacher (45). Methoxyhydroxychlor,(IV) (0.6g.) was refluxed in 3N methanolic potassium hydroxide (50ml.) for 0.5hr.. The reaction mixture was added to cold water (150ml.) and acidified. The precipitated product (0.34g.) was filtered and recrystallized

from cyclohexane giving white fluffy crystals, m.p. 120-122° [Lit.110-115°(1)]. The NMR spectrum **(C_2D_6CO) contained signals at τ7.15 (1H,-OH), τ6.17 (3H,-OCH_3) and τ2.6-3.3 (8H). The mass spectrum contained M^+ at *m/e* 294 (100%, $C_{15}H_{12}Cl_2O_2^+$) and a major fragment at *m/e* 224 (58%, $C_{15}H_{12}O_2^+$).

Analysis: Calcd. for $C_{15}H_{12}Cl_2O_2$: C,61.04; H,4.10; Found: C,61.78; H,4.24%.

2,2-Bis (*p* - methoxyphenyl)-1,1- dichloroethane, (Methoxydichloromethyl derivative,VIII), was prepared according to the method of Zepp *et. al.* (12). A mixture of dichloroacetal (4.0g.) and anisole (4.2g.) was added to dry chloroform (100ml.) with cooling in an ice bath. Anhydrous aluminium trichloride (5.4g.) was slowly added to the mixture with continuous stirring in nitrogen, after which stirring was continued for a further 0.5hr. at 0° and another 16hr. at room temperature. The reaction mixture was poured into water and the chloroform layer collected. The aqueous layer was extracted with ethyl acetate (150ml.) and this was added to the chloroform extract. The combined organic extract was washed, dried (Na_2SO_4) and the solvent removed, to yield a crude product (2.4g.) from which excess anisole was removed by distillation under reduced pressure. Recrystallization from a benzene-ethanol mixture gave

the pure product (0.18g.) which had m.p. 112-113° [Lit.116-117°(12)]. The NMR spectrum ** ($CDCl_3$) contained signals at τ7.7 (6H,2-OCH_3), τ7.0 (α-H, doublet), τ5.2 (-$CHCl_2$, doublet) and at τ4.2-4.75 (8H). The mass spectrum contained M^+ at *m/e* 310 (7%, $C_{16}H_{16}Cl_2O_2^+$) with the base peak at *m/e* 227 (100%, $C_{15}H_{15}O_2^+$).

Analysis: Calcd. for $C_{16}H_{16}Cl_2O_2$: C,61.75; H,5.18; Found: C,61.49; H,5.26%.

2,2-Bis (*p* - hydroxyphenyl)-1,1- dichloroethane, (Hydroxydichloromethyl derivative,IX), was synthesized similar to the dichloromethyl derivative,(VIII). Thus, phenol (6.0g.) and dichloroacetal (6.0g.) were dissolved in dry chloroform (150ml.) at 0-5°. Anhydrous aluminium trichloride (9.0g.) was slowly added with continuous stirring in nitrogen and the resulting reaction mixture was stirred for 1hr. at 0°. Stirring was continued for 16hr. at room temperature, then the reaction mixture was poured into water and the chloroform layer was collected. The aqueous layer was filtered giving some crude product and the filtrate was extracted with ethyl acetate (150ml.). The organic extracts were washed with water, dried (Na_2SO_4) and the solvent removed, leaving a residue (4.17g.) which was recrystallized from a benzene-ethanol mixture, to give the desired product (1.2g.) which had m.p. 178-179°. The NMR spectrum **

(C_2D_6CO) contained signals at τ7.01 (2H,2-OH), τ6.5 (α-H, doublet) and τ2.6-3.35 (8H). The signal for the ($-CHCl_2$) proton was masked beneath that of the aromatic protons and its presence was confirmed by spin decoupling as discussed elsewhere (see p.49). The mass spectrum contained M^+ at *m/e* 282 (8%, $C_{14}H_{12}Cl_2O_2^+$) with the base peak at *m/e* 199 (100%, $C_{13}H_{11}O_2^+$).

Analysis: Calcd. for $C_{14}H_{12}Cl_2O_2$: C,59.59; H,4.25; Found: C,59.30; H,4.38%.

4,4'-Dihydroxybenzophenone,(XIV) was obtained from Aldrich Chemical Co. (97% pure) and had m.p. 213-215°. It was used without further purification. Its NMR spectrum ** (C_2D_6CO) contained signals at τ6.9 (2H,2-OH) and at τ2.15-3.1 (8H). The mass spectrum contained M^+ at *m/e* 214 (36%, $C_{13}H_{10}O_3^+$) with the base peak at *m/e* 121 (100%, $C_7H_5O_2^+$).

Attempted preparation of 2,2-bis (*p* - hydroxyphenyl) -1- monochloroethylene, (Hydroxymonochloro olefin,XIX). The method was similar to that reported for the preparation of the methoxy counterpart (19). Thus, the dihydroxydichloromethyl compound,(IX, 0.5g.) was refluxed in 50ml. of ethanol containing potassium hydroxide (1.0g.) for 1.5hr.. The resulting reaction mixture was added to cold distilled water (50ml.), acidified and extracted with ethyl acetate (150ml.). The organic extract was dried (Na_2SO_4), the

solvent removed and the crude product (0.44g.) was recrystallized from a methanol-benzene mixture. The m.p. was 220-222° and the NMR spectrum ** (C_2D_6CO) had signals at τ2.55-3.35 (8H) and at τ7.05 (OH)ppm. Signals at τ5.8, τ6.6-7.0 and τ9.0ppm could not be assigned. The mass spectrum, which did not show the presence of chlorine, contained M^+ at *m/e* 256 with major fragments at *m/e* 212, *m/e* 151 and *m/e* 123. The molecular ion corresponds to $C_{16}H_{16}O_3^+$ with precise mass calculated *m/e* 256.1099 and found *m/e* 256.1032.

Analysis: Calcd. for $C_{16}H_{16}O_3$: C,74.96; H,6.25; Found: C,74.82; H,6.40%.

The desired product $C_{14}H_{11}O_2Cl$ should have M^+ at 246 and analysis C,68.29; H,4.47%.

(iii) Metabolism of Methoxychlor by *Klebsiella pneumoniae*

Triplicate cultures of the bacterium *K.pneumoniae* (formerly *A.aerogenes*, ATCC no. 13883) were grown aerobically in 3% trypticase soy broth (100ml.) to which the appropriate amounts of the various test compounds were added in acetone (0.1ml.). Incubation was at 37° for periods of time ranging from 3 to 8 days. Control cultures were grown simultaneously under identical conditions and without addition of chemicals. After incubation, all cultures were autoclaved and the appropriate amounts of the test compounds were added

to the controls which were allowed to stand for 1.5-3hr. at room temperature. The cells in each culture were disrupted (29) with a sonifier Model W-350 cell disruptor obtained from Branson Sonic Power Co. fitted with a micro-tip and set at an output level of 6.5 in the continuous operating pulse mode, for 5min.. Each culture was then extracted with ethyl acetate (3 x 50ml.), the combined extracts of each culture were dried (Na_2SO_4) and the solvent removed. The resulting residues were dissolved in ethyl acetate (10ml.) and applied to a small column (1cm., i.d.) containing 2.0g. Silicagel. The column was further eluted with ether-pet. ether (30-60°) (1:9), 15ml. for all dimethoxy compounds and 25ml. for all hydroxy compounds. The eluant from each culture was collected, concentrated to 5.0ml. (nitrogen stream), transferred to screw cap vials fitted with rubber-teflon septums and the remaining solvent was evaporated. Preliminary identifications of residual test compounds and possible metabolites were obtained using thin layer chromatography (see p.107). Addition of 1.0ml. of an appropriate internal standard solution to each vial provided for quantitative gas chromatography (see p.108). Methoxychlor, its dichloromethyl derivative, (VIII) and methoxychlor olefin, (V) were analysed with

a SE-30(5%) column with ethoxychlor,(II) as internal standard (2.0mg./ml.) at a column temperature of 230°. The hydroxy derivatives of methoxychlor were analysed, after silylation, using both the SE-30(5%) and Dexsil 300 (3%) columns (see p.73). DDT was the internal standard (2.5mg./ml.) when only residual hydroxychlor, (III) was measured and the column temperature was 250°. For samples containing hydroxychlor,(III) and hydroxychlor olefin,(VI) and/or the dihydroxydichloromethyl derivative, (IX), methoxychlor olefin,(V) was used as an internal standard (1.0mg./ml.) and the column temperature was 220° (for the structures of methoxychlor and its derivatives mentioned above, see p.24). Calibration curves for each compound were determined immediately prior to the actual analyses.

Since all cultures were grown in triplicate, the data provided in Table 3 (see p.70) are consequently the averages of three analyses.

(iv) Fungitoxicity

The fungi used were *Mortierella pusilla* (Oudemans), provided by Dr. G. Harvais, Department of Biology, Lakehead University, *Mortierella isabellina* (Oudemans) and *Trichoderma viride* (Persoon), obtained from Dr. J.P. Kutney, Department of Chemistry, University of British Columbia, and *Saprolegnia parasitica* (Coker)

was ATCC no. 22284.

Fungitoxicity was measured by the plate culture bio-assay method (21). The fungi were grown on a modified malt agar medium consisting of, Dextrose,(7g.), Casamino acids,(1g.), Yeast Extract,(0.5g.) and Agar, (15g., USP) per litre of distilled water. In addition to these ingredients the medium for *T.viride* contained one percent (1%) cellulose powder. The media contained logarithmically increasing dosages of the compounds used, and the correct amount of each was added in acetone (1.0ml.) to 75ml. of hot agar medium immediately after it was autoclaved (15min. at 15p.s.i.) so that most of the solvent would evaporate. From these solutions, plates were poured in triplicate for each dose level. Control plates, in triplicate, were poured from agar solutions to which only acetone (1.0ml.) had been added. Each plate was inoculated at the centre with a 3mm. diameter standard inoculum cut from the actively growing front of previous plate cultures. For plates grown with *S.parasitica*, the inocula were 5 x 5mm. squares, cut with a sterile surgical scalpel. When the controls had grown almost to the full extent of the Petri-dish, (approximately 7.5cm., 3 to 4 days for *S.parasitica*, 4 to 6 days for *T.viride* and 13 to 16 days for the *Mortierella* species) colony diameters were measured. The degree

of inhibition of mycelial growth was calculated from the mean differences between the controls and treated plates, and expressed as a percentage of the former. Dosage response curves were obtained by plotting percentage inhibition on a probit scale (61) against pesticide concentration on a log. scale. Pesticide concentrations ranged from (1.6-400)ppm for all species except *S.parasitica*, for which an extended concentration range of (0.2-400)ppm was used so as to accommodate the higher sensitivity of this species towards some of the compounds.

The nature of the inhibiting effect was established for two of the most toxic compounds as follows. *T.viride* and the two *Mortierella* species were grown in liquid nutrient medium (100ml.) containing dextrose,(5g.), asparagine,(2g.), potassium dihydrogen orthophosphate, (KH_2PO_4, 1g.), magnesium sulphate,($MgSO_4.7H_2O$, 0.5g.), vitamins stock,(0.2ml.), (see Appendix I) and minerals stock,(2.5ml.), (see Appendix I) per litre of distilled water. The medium for *S.parasitica* contained Casamino acids,(1g.) and Yeast Extract,(0.5g.) instead of asparagine as growth was very slow when the latter component was used. Triplicate media, at three dose levels [(50, 100, and 200ppm) for each compound (III and IX), added to the hot medium in acetone (0.1ml.)

immediately after autoclaving (15min. at 15p.s.i.)], were inoculated with inocula (5 x 5mm. for *S.parasitica* and 10 x 10mm. for the other three fungi), cut from the active growing front of previous plate cultures after the medium had cooled to room temperature. The resulting mixtures in 250ml. Erlenmeyer flasks were shaken on a rotary table shaker (stroke, 12cm., 90r.p.m.) at room temperature for periods of time which produced normal growth in a set of controls. Normal growth (in the absence of the compounds) was represented by the average dry weight of the filtered mycelium of a mature culture (59). From flasks in which no growth was observed after this incubation period, the inocula were removed, rinsed throughly with distilled water and placed on pesticide free agar plates. Normal colony growth was observed on the plates for the two *Mortierella* species and *T.viride*, but there was no growth on the plates with the inocula of *S.parasitica*.

(v) Fungal Metabolism

Liquid cultures (100 or 200ml.) prepared and inoculated as before (p.123) were grown in triplicate after the addition of methoxychlor (from 10-20ppm) in acetone (0.1ml.), for periods of time varying from 6 days to 3 weeks on a rotary table shaker (stroke, 12 cm., 90r.p.m.). With each triplicate set, a set of

control cultures, to which only acetone (0.1ml.) was added, was grown under the same conditions. At the end of the fermentation period, each culture was blended in a Waring Blendor (1min.) and extracted with ethyl acetate (3 x 50ml. for 100ml. cultures and 2 x 100ml. for 200ml. cultures). The combined extracts from each culture were dried (Na_2SO_4) the solvent removed, and the remaining residue was dissolved in ethyl acetate (1ml.). The resulting solutions from each culture were subjected to thin layer chromatography (TLC). When any known methoxychlor metabolites (as discussed in Chapter 2) were found to be present in the extracts, their identities were confirmed by gas chromatography, if necessary after silylation as described (p.108). Such identifications were confirmed on at least two different stationary phases.

For the accumulation of an unknown metabolite of methoxychlor observed in extracts of *T.viride*, a total of thirty-six liquid cultures (200ml. each) containing 20ppm of methoxychlor were inoculated and grown for three weeks. In this series, the mycelial masses were filtered, blended and extracted. The aqueous parts of these cultures were similarly extracted. A total combined residue (0.766g.) was subjected to column chromatography (Silicagel 10g., column i.d., 2cm.).

Elution with ether-hexane (3:1) gave the metabolite (35mg.) which was further purified by preparative thin layer chromatography on 20 x 20cm. plates (Whatman, PLKF, 1000μ thickness) which were eluted with ether-hexane (3:1). The pure compound had m.p. 172-174° and M^+ at *m/e* 428. The mass spectrum did not indicate the presence of chlorine, and the NMR spectrum ** ($CDCl_3$) contained signals which showed the presence of *p* - substituted aromatic rings and a large number of aliphatic protons.

APPENDIX I, Recipes for Growth Media.

Minerals Stock Solution

$FeCl_3.6H_2O$	98 mg.
$CuSO_4.5H_2O$	78.5 mg.
$MnSO_4.4H_2O$	40.5 mg.
$ZnSO_4.7H_2O$	88 mg.
Distilled water to	250 ml.

Vitamins Stock Solution

Biotin	25 µg.
Thiamine	500 µg.
Pyridoxine	500 µg.
Inositol	25 µg.
Ethyl alcohol (40%) to	100 ml.

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