6-1967

Metabolic Pathways for Methoxychlor in Rats

Frank Hastings McKim
Union College - Schenectady, NY

Follow this and additional works at: https://digitalworks.union.edu/theses

Part of the Chemistry Commons

Recommended Citation
https://digitalworks.union.edu/theses/1980

This Open Access is brought to you for free and open access by the Student Work at Union | Digital Works. It has been accepted for inclusion in Honors Theses by an authorized administrator of Union | Digital Works. For more information, please contact digitalworks@union.edu.
METABOLIC PATHWAYS FOR
METHOXYPHLOXOR IN RATS

by

Frank Hastings McKim

Senior Thesis Submitted
in Partial Fulfillment
of the Requirements of Graduation

DEPARTMENT OF CHEMISTRY

UNION COLLEGE

MAY 1967
This Thesis

Submitted by

Frank H. McKinn

to the

Department of Chemistry Union College

in partial fulfillment of the requirements of the degree of

Bachelor of Science with a Major in Chemistry

is approved by

David M. Xenone
ACKNOWLEDGEMENT

The author wishes to express his gratitude and appreciation to Dr. David M. Serrone whose guidance was invaluable for the completion of this project. Appreciation is also extended to the Institute of Experimental Pathology and Toxicology at Albany Medical College for the use of their facilities and their support of this project.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES AND GRAPHS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHOXYCHLOR AND ITS METABOLIC IMPORTANCE</td>
<td>1</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>2</td>
</tr>
<tr>
<td>CHEMICAL AND THEORETICAL ASPECTS OF METHOXYCHLOR</td>
<td>4</td>
</tr>
<tr>
<td>EXPERIMENTAL APPROACH AND METHOD</td>
<td>13</td>
</tr>
<tr>
<td>METABOLIC RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>SUGGESTIONS FOR FURTHER STUDY</td>
<td>34</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>38</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND GRAPHS

TABLE I  Methoxychlor metabolism in female rats  20
FIGURE I  Methoxychlor-one hour time study with various concentrations  21
FIGURE II  Methoxychlor time study  22
FIGURE III  Methoxychlor standard infrared spectra  24
FIGURE IV  Incubated sample of methoxychlor-extracted with ether from thin layer.  25
FIGURE V  Methoxychlor metabolites-infrared spectra  26
FIGURE VI  Methoxychlor metabolites-infrared spectra  27
TABLE II  $R_f$ values of experimental metabolites  28
TABLE III  $R_f$ values of theoretical metabolites  28
TABLE IV  $R_f$ values of bile, liver and incubate extracts  30
TABLE V  $R_f$ values for standards  30
TABLE VI  Experimental metabolites from incubated methoxychlor. Retention times of major peaks.  31
TABLE VII  Theoretical metabolites-retention times for major peaks.  31
TABLE VIII  Retention times of experimental metabolites from an incubated sample of methoxychlor.  32
TABLE IX  Theoretical metabolites-retention time for major peaks.  32
TABLE X  Experimental metabolites from an incubated sample of methoxychlor and ether extracts of liver and bile from rats treated with methoxychlor.  33
METHOXYPHTHAL AND ITS METABOLIC IMPORTANCE

Methoxychlor was one of the chlorinated hydrocarbon compounds developed after World War II. Geigy and DuPont hold patents on methoxychlor and they were the groups that discovered the insecticidal properties of the compound. Although relatively non-toxic to mammals (LD₅₀ for rats is between 5000 and 6000 mg. per kg. orally), methoxychlor is effective as an insecticide for farm produce and livestock. Methoxychlor is metabolized rather rapidly and therefore is not readily stored in body fat. Due to this property methoxychlor is preferred over DDT which is stored in body fat.

Widespread use of methoxychlor has lead to increasing amounts of its residues on farm produce and in waterways. New analytical techniques have led to more accurate analysis of these residues. The extensive use of methoxychlor and rising residue levels of the pesticide have caused some concern as to the effects produced in animals when they consume methoxychlor. One part of this research should be on the metabolic pathway followed when methoxychlor is detoxified.

The toxic effects of methoxychlor on rats and monkeys have been recently investigated at Albany Medical College. A higher dose of methoxychlor is required to obtain similar toxicologic effects as observed with DDT. This information becomes important when considering an approach to methoxychlor metabolism.
The medical and biological importance of knowing how methoxychlor is metabolized should be apparent in light of other pesticide research. In many instances, it is not the initial drug, but rather a metabolite which causes the physiological action on the body. If it is determined that a metabolite of methoxychlor causes a toxic reaction rather than the primary compound, then further clinical research can be done with the secondary product instead of methoxychlor.

To determine the validity of these arguments, the metabolites must be isolated and identified. Animals must then be treated with the isolated metabolites to determine whether or not they have an effect.

There is also a commercial reason for determining the metabolites of methoxychlor. As mentioned above, a secondary metabolite might be the active pesticidal agent. If this is true then the manufacturer can produce the active agent directly and avoid losing part of his product in other reactions of the body. Hopefully he could then make his product cheaper.

**SUMMARY**

The research status of methoxychlor has so far been discouraging. Apparently the high number of probable metabolites will make identification a long tedious task. The main approach of the experiment was to metabolize methoxychlor with rat liver microsomes since hepatic microsomes
have been shown to contain many of the metabolic enzyme systems. The incubated sample was extracted with various solvents after hydrolysis. The extracts were separated and identified by thin layer or vapor phase chromatography. The structures of the metabolites were theoretically determined by studying the analogy between DDT and methoxychlor. A study of detoxification mechanisms also aided in establishing a theoretical metabolic chain.

The next step was to compare the actual metabolites from an incubated methoxychlor-enzyme system with the theoretical standards. In several instances the proposed standards had to be chemically prepared. Methods are given in the experimental procedures for these preparations. Unfortunately not all the necessary standards are available. Further research will be necessary to prepare the remaining theoretical metabolites. Results are still inconclusive as a result of this lack of standards.
CHEMICAL AND THEORETICAL ASPECTS OF METHOXYCHLOR

Considerable research has been done with methoxychlor. However little study on the metabolism of the compound has been performed. Most research has dealt with pesticidal properties, residue techniques or analytical methods. Some information has been gathered about physical properties of methoxychlor and its relation to toxicology.

The molecular weight of methoxychlor is 346 and the structure is shown below:

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{H} \\
\text{C} & \quad \text{OCH}_3 \\
\text{Cl} \quad \text{C} \quad \text{Cl} & \\
\text{Cl} 
\end{align*}
\]

The compound normally exists as a white crystalline compound or powder with a melting point of 83-86° (2). In commercial use it is generally formulated as a dust or spray with varying percentages of methoxychlor. Most commercial preparations are technical, but a fairly pure methoxychlor can be recrystallized from ethanol.

Residue analysis has become important since methoxychlor has seen widespread use. Techniques have now been developed allowing a determination of methoxychlor in quantities as small as parts per million. Along with this residue analysis, analytical techniques have been developed. Electrochemical methods are popular, but chemical methods such as vapor phase
chromatography and thin layer analysis are generally more applicable to laboratory situations (3,4). In metabolic work, thin layer, vapor phase, and infrared analysis are most useful. If available, radiochemical investigation is helpful. The analytical techniques available for methoxychlor should be useful in identifying the metabolites.

The production of methoxychlor can be performed in the laboratory by combining two moles of anisole with chloral hydrate. This reaction is shown below:

\[
\begin{align*}
2 \text{CH}_3_2_0_+ & \overset{H_2SO_4}{\longrightarrow} \text{CH}_2_0_+ \overset{C_1-C-C}{\longrightarrow} \text{CH}_2_0_+ \overset{C_1-C-C}{\longrightarrow} \text{CH}_2_0_+ \overset{C_1-C-C}{\longrightarrow} \text{CH}_2_0_+
\end{align*}
\]

This method can lead to some impurities since the anisyl ring can be attacked at positions other than para. However the para attack is most likely since the ring would be activated in that position.

Actual research dealing with methoxychlor metabolism has shown little information of great interest except for a paper by Weikel (5). This work was done primarily with radio labeled methoxychlor. Weikel's approach was to attempt a comparative study of DDT and methoxychlor.
The chemical similarity between DDT and methoxychlor is rather useful in attempting to theorize a metabolic pathway. The structure of DDT is:

\[
\begin{array}{c}
\text{Cl} \quad \text{H} \\
\text{C} \quad \text{C} \\
\text{Cl} \quad \text{Cl} \\
\end{array}
\]

M.W. = 355  
M.P. = 110

The structural similarity shows why methoxychlor might be expected to undergo metabolism in a fashion similar to DDT. The one primary chemical difference between DDT and methoxychlor is that the chlorine on the ring is chemically deactivating to further reaction on the ring whereas the methoxy group on the phenyl ring of methoxychlor is activating. The information might be useful in predicting biological intermediates.

Research on DDT has been rather extensive. Many of the metabolites have been identified and confirmed. Initial work by White and Sweeney in 1945 (6) indicated that bis-(p-chlorophenyl) acetic acid was the major metabolite. They also theorized that 1,1 dichloro- 2,2 bis (p-chlorophenyl) ethylene was an intermediate metabolite. Work has shown that:
Peterson and Robison (7) went further with the research and added several more metabolites to the dosier on DDT. The proposed scheme of DDT metabolism is as follows. Several of these compounds were isolated and identified.
The metabolites were identified by treating rats with DDT, sacrificing them and then extracting the tissue samples. The samples were then partitioned in column chromatography. Identification was made through the use of thin layer chromatography.

Weikel's research can now be evaluated. The excretion of DDT is primarily in the urine. However Weikel found that radioactivity was found in the bile only minutes after an
intravenous injection of methoxychlor. Final excretion was in the feces. Weikel made no attempt to identify the structure of the metabolites, but he did find through thin layer analysis that the metabolites consist of strongly anionic compounds which are rather water soluble. Following the DDT analogy here, led us to believe that an acidic compound could well be the identity of Weikel's highly anionic water soluble compound. The suspected identity of this compound will be explained after a brief summary of other possible detoxification mechanisms of methoxychlor.

In the case of methoxychlor there are two O-CH₃ groups on each end of the compound. Williams (8), in *Detoxification Mechanisms*, suggests that methoxychlor is demethylated. He draws this conclusion from the fact that strains of flies have been developed which are resistant to methoxychlor but not DDT. Metabolism of DDT occurs through dehalogenation. Also of coincidence here is the result that DDT resistant flies also have some tolerance to methoxychlor, indicating that both insecticides have similar modes of detoxification. Williams goes further in discussing metabolism of ethers, to say that the likely demethylated product of a substituted anisole could be a conjugated phenol.

An easy way to test the demethylase theory of methoxychlor metabolism is to test methoxychlor in a standard demethylase system. This would involve incubation with hepatic microsomes.
Such a test was designed for methoxychlor and results indicated that methoxychlor is rapidly demethylated.

When the two modes of detoxification are combined, we can theorize a complete metabolic pathway for methoxychlor. The main points of DDT metabolism are dehalogenation and eventual conversion to an acid. Methoxychlor can undergo this mode as well as being demethylated. The theoretical pathway of methoxychlor is therefore shown below:

**Methoxychlor**

\[
\begin{align*}
\text{(A)} & \quad \text{CH}_3\text{O} & \quad \text{Cl} - \text{C} - \text{C} - \text{Cl} \\
\text{H} & \quad \text{Cl} & \\
\end{align*}
\]

\[
\begin{align*}
\text{(B)} & \quad \text{OCH}_3 & \quad \text{C} - \text{C} - \text{Cl} \\
\text{H} & \quad \text{Cl} & \\
\end{align*}
\]

\[
\begin{align*}
\text{(C)} & \quad \text{Cl} & \quad \text{Cl} \\
\end{align*}
\]

This pathway assumes that demethylation occurs before dehalogenation. If dehalogenation occurs first then we could also expect the following metabolites to be significant:
These are the metabolites that would be considered to exist in measurable quantities. Of course each step might involve several intermediates, but these would be difficult to determine. Partial metabolism could easily lead to unsymmetrical products with the O-CH$_3$ group on one end and with the conjugate on the ether end. The conjugate (con) could probably be broken down with acid hydrolysis for analytical purposes.

The theoretical metabolic scheme set forth above is the one basically tested for in the project. The major approach was to obtain through preparation or purchase the theoretical compounds for standards. These compounds were then compared with the actual metabolites isolated from an incubated sample.

The major criticism of the above hypothesis is that it limits metabolism to one basic pathway. In the case of methoxychlor, several different pathways are possible for the metabolism of the anisole ring. Williams suggests that aromatic ethers can be detoxified by hydroxylation of the ring, or by hydroxylation of the ring and demethylation of the ether, or by simple demethylation as already described.
To try to determine which pathway would be followed is difficult to determine even with the guidelines set forth in *Detoxification Mechanisms*. However when methoxychlor is incubated in the demethylase system, there is evidence to show that demethylation occurs rapidly. Evidence for this appears in the results.

The question of detoxification of the tri-chloro group has been fairly well settled by DDT research. This mechanism is therefore not in question as much as the detoxification of the anisole ring. It must be remembered in this experiment that the biological degradations can occur by many different methods. It is also possible for any one particular intermediate in the reaction to be the principal metabolite. Therefore, the proposed metabolic chain could be accurate, but the emphasized metabolites may not be the major metabolites.
EXPERIMENTAL APPROACH AND METHOD

The approach and method taken in determining the metabolites of methoxychlor has already been described. Basically the method involved incubating methoxychlor with an appropriate enzyme system. The metabolites were then separated and compared to theoretical standards.

Incubation Method:
Materials: 0.3m phosphate buffer (ph 7.4), MgCl₂ (25 umoles), nicotinamide (100 umoles), glucose-6-phosphate (100 umoles), TPN (0.3 umoles), male rat liver microsomes (from 200 mg. of liver), methoxychlor (10 umoles) in ethanol and hydrochloric acid used for hydrolysis. The amounts given above are for a single sample. When the samples were used for extraction, these values were multiplied by the desired factor. A Dubnoff metabolic incubator was used at 37°C for incubation.

The mixture is metabolized for varying lengths of time at 37°C. After incubation, the incubate is hydrolyzed with hydrochloric acid for 15 minutes in boiling water. The length of time, and water temperature used in hydrolysis seemed to make little difference. Apparently hydrolysis of the conjugate occurs rapidly. If an assay is to be run for demethylase activity, semicarbazid must be added to the incubation mixture. Demethylase activity is measured by formaldehyde production as determined by the Nash reaction.
Extraction Procedure:

Several different extraction procedures were used on the hydrolyzed samples. At first chloroform : methanol (2:1) was used for extraction. It was later found that several of the theoretical metabolites were insoluble in chloroform. Ether was considered to be a more efficient solvent since all the theoretical metabolites are soluble. The sample was extracted several times with ether and concentrated by evaporation.

Analytical Techniques:

Materials: Perkin and Elmer programmed temperature gas chromatograph equipped with a glass injector block and column. A flame detector was utilized. The column was packed with Dow Corning Compound 200. P and E Infracord 137 Dual Beam Infrared spectrophotometer was used for IR spectra. Thin layer apparatus consisted of Eastman Kodak plastic sheets coated with silica gel. The usual mobile solvent phase was hexane : ether : acetic acid (120 : 35 : 5). Various detection systems were utilized for TLC work. These included silver nitrate, diphenylamine and zinc chloride reagent, rhodamine blue and brom cresol green.

The ether extracts of the incubate were chromatographed on the Eastman plastic sheets or standard glass plates with a layer of silica gel. Various detection reagents were utilized to determine Rf values of the metabolites. In nearly all cases standard methoxychlor was chromatographed with the samples.
Blanks were prepared from an incubated sample without methoxychlor. The blank was extracted with ether and this extract was used as a check for the samples.

The first approach was to try to elute the separate bands of metabolites produced by TLC. The eluted samples were then run on the infrared spectrometer. This method was designed to identify various structural groups among the metabolites. This method was found to be relatively inefficient except for the identification of unmetabolized methoxychlor. A spectra of methoxychlor was obtained from the TLC plate at an Rf value between 0.5 and 0.7. This spectra compared very favorably with the methoxychlor standard run in carbon disulfide or carbon tetrachloride. In all cases the eluted metabolites were evaporated to dryness and redissolved in carbon tetrachloride or carbon disulfide. This technique would be quite valuable and will be re-examined if sufficient amounts of metabolites can be recovered from the incubated samples. There have been difficulties in obtaining enough of any one metabolite for a respectable infrared spectra.

The technique was then modified to be used in conjunction with a vapor phase chromatograph. The standards and metabolites were run concurrently with TLC and vapor phase chromatography.
Attempts were made to correlate Rf values and retention times of actual metabolites and the theoretical standards. This method should allow confirmation of the identity of the metabolites.

An attempt was also made to use ultraviolet analysis in the work. Unfortunately a trial run indicated that it would be difficult to distinguish one peak from another.

Preparations:

Several of the theoretical standards were not commercially available and had to be prepared in the laboratory. In the cases of compounds B, C, and D, we prepared a hydroxy form since it was assumed that the conjugated metabolites were broken down by acid hydrolysis.

1,1 dichloro - 2,2 bis (p-anisyl) ethylene (E): Methoxychlor was used as a starting material and dehydrohalogenated with 3N methanolic potassium hydroxide.

Methoxychlor (0.2 mole) was refluxed 1 hr. with 200 ml. 3N methanolic KOH. The resulting compound was recrystallized with 600 ml. ethanol. Melting point was 109°-112° (9).

1,1,1 - trichloro - 2,2 - bis (p-hydroxyphenyl) ethane (B): Made by combining phenol with chloral hydrate according to the method of Hubacher.

The reaction mixture was not easily separated. A crystalline compound was finally isolated though with a melting point between 195° - 200°C. However, with TLC, a streak was obtained instead of a clean spot.
It was assumed that an impure preparation of the compound was obtained. Its melting point did correlate with reported results (199-204) (9).

1,1 dichloro - 2,2 bis (p-hydroxyphenyl) ethylene (C):
Compound B (Above) was treated with potassium hydroxide. This method also was taken from Hubacher's work. Once again an impure product was obtained since the starting compound was impure. The experimental melting point did compare to Hubacher's reported value (210-213 dec).

bis (p-hydroxyphenyl) acetic acid (D):
This was prepared by condensing glyoxylic acid with phenol. Technical glyoxylic acid was used due to its availability.

The reaction mixture contained 40.3 ml glyoxylic acid (40%) 7 ml sulfuric acid, 37.6 gm. phenol and 3 ml of acetic acid. The mixture was maintained at 50°C for 5 hours. The mixture is allowed to sit for two days then dissolved in 100 ml water. This mixture is extracted with ether. The ether extract is then washed with sodium carbonate.

After evaporating the ether, the oily liquid is shaken with benzene. The resulting crystals are then purified by dissolving them in a large volume of 1,2 dichloroethane. The impure glyoxylic acid apparently caused some side reactions, since the final product was impure, although its melting point corresponded to the reported value 146-151° (9).
bis (p-anisyl) acetic acid (F):

An attempt was made to synthesize this compound with the technical glyoxylic acid, but a crude product was obtained. Purification was not attempted though since Hubacher was able to graciously supply us with a sample.

This covers the major metabolites theorized.
METABOLIC RESULTS

Metabolic studies were performed on methoxychlor by incubating the compound in a hepatic microsomal enzyme preparation. In order to determine if methoxychlor was metabolized by this system, several trials were made to determine formaldehyde production in the incubated sample. This was done by including semicarbazide in the incubation mixture to trap the formaldehyde as the semicarbazone.

The final analysis was by the Nash reaction. Formaldehyde standards had previously been run for this test in routine enzyme work at Albany Medical College.

For methoxychlor it must be remembered that there are two possible sites for 0-demethylation. We could expect two moles of formaldehyde for every mole of methoxychlor completely demethylated. The demethylation results for male rats are shown in Figures I and II. Figure I is a plot of formaldehyde production against methoxychlor concentration in the sample. From the graph it is apparent that in concentrations of less than 5 umoles per gram liver, methoxychlor is almost completely metabolized by demethylation in one hour. At higher concentrations, little of the excess methoxychlor is metabolized, indicating substrate saturation of the enzyme. Generally the incubated samples used for thin layer chromatography contained 50 umoles of methoxychlor per gram liver. This insured that...
some free methoxychlor would be recovered as a check on the experimental techniques.

The second graph (Fig. II) is a study of formaldehyde production versus time of incubation. The reaction appears to start out rapidly and then the rate slowly drops until it is constant. This slowing effect could be due to exhaustion of essential components of the enzyme system or product inhibition.

Female rat livers gave entirely different results with demethylation studies. In all such tests the female hepatic microsomes had little or no demethylase activity as compared to the males. It has been previously established that female rats possess little hepatic microsomal enzyme in comparison to male rats.

<table>
<thead>
<tr>
<th>Methoxychlor concentration</th>
<th>Formaldehyde produced at</th>
</tr>
</thead>
<tbody>
<tr>
<td>umoles/gm.liver</td>
<td>15 min. 30 min. 45 min. 60 min.</td>
</tr>
<tr>
<td>2.5</td>
<td>0.625 0.750 0.720 0.625</td>
</tr>
<tr>
<td>12.5</td>
<td>0.820 0.750 0.750 0.720</td>
</tr>
<tr>
<td></td>
<td>umoles/gm liver</td>
</tr>
</tbody>
</table>
Recovery of the metabolites after incubation was unfortunately not clean enough in most cases to obtain even a fair infrared spectra. Some incubated samples which contained a large excess of methoxychlor did yield clear spectra of that compound after thin layer chromatography and subsequent elution. In figure III a spectra of methoxychlor in carbon disulfide is shown. In comparison with figure IV, which is a spectra of a compound seperated from ether extract of an incubation sample of methoxychlor, we find very good correlation to the standard. This does indicate that the method of incubation, extraction, TLC and eventual infrared identification is useful if high enough concentrations of the metabolite can be obtained. Several of the other spectra of fractions from a thin layer plate analysis of methoxychlor metabolites are presented. None of these spectra are sharp enough to predict the groups present. On each spectra r_f values are given for the fraction of the thin layer plate used.

Thin layer chromatography was used to compared the actual metabolites to the theoretical standards as they became available. The usual solvent system used was hexane:ether:acetic acid (120:35:5). Several different detection reagents were used on the plates (11,12). The reagents most often utilized were silver nitrate in ethanol, diphenylamine and zinc chloride in acetone, rhodamine B in ethanol, brom cresol green, and formazlin in sulfuric acid.
<table>
<thead>
<tr>
<th>SAMPLE NO.</th>
<th>LENGTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>1</td>
</tr>
</tbody>
</table>

**LEGEND**
- 1. Reagent
- 2. Extracted

**LEGEND**
- 1. Reagent
- 2. Extracted

**REMARKS**
- DATE: Aug. 19, 1966
- OPERATOR: McCon

**PHASE NO.**
- 0.1

**THICKNESS**
- 0.1

**OBJECT**
- Chlo.: HCl.

**SAMPLE**
- 3. Preparation

**ORIGIN**
- 2. Preparative

**WAVELENGTH (MICRONS)**
- 0 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

**SPECTRUM NO. 1**
- 1.1

**SPECTRUM NO. 2**
- 1.2

**SPECTRUM NO. 3**
- 1.3

**SPECTRUM NO. 4**
- 1.4

**SPECTRUM NO. 5**
- 1.5

**SPECTRUM NO. 6**
- 1.6

**SPECTRUM NO. 7**
- 1.7

**SPECTRUM NO. 8**
- 1.8

**SPECTRUM NO. 9**
- 1.9

**SPECTRUM NO. 10**
- 1.10

**SPECTRUM NO. 11**
- 1.11

**SPECTRUM NO. 12**
- 1.12

**SPECTRUM NO. 13**
- 1.13

**SPECTRUM NO. 14**
- 1.14

**SPECTRUM NO. 15**
- 1.15

**SPECTRUM NO. 16**
- 1.16

**SPECTRUM NO. 17**
- 1.17

**SPECTRUM NO. 18**
- 1.18

**SPECTRUM NO. 19**
- 1.19

**SPECTRUM NO. 20**
- 1.20

**SPECTRUM NO. 21**
- 1.21

**SPECTRUM NO. 22**
- 1.22

**SPECTRUM NO. 23**
- 1.23

**SPECTRUM NO. 24**
- 1.24

**SPECTRUM NO. 25**
- 1.25

**SPECTRUM NO. 26**
- 1.26

**SPECTRUM NO. 27**
- 1.27

**SPECTRUM NO. 28**
- 1.28

**SPECTRUM NO. 29**
- 1.29

**SPECTRUM NO. 30**
- 1.30

**SPECTRUM NO. 31**
- 1.31

**SPECTRUM NO. 32**
- 1.32

**SPECTRUM NO. 33**
- 1.33

**SPECTRUM NO. 34**
- 1.34

**SPECTRUM NO. 35**
- 1.35

**SPECTRUM NO. 36**
- 1.36

**SPECTRUM NO. 37**
- 1.37

**SPECTRUM NO. 38**
- 1.38

**SPECTRUM NO. 39**
- 1.39

**SPECTRUM NO. 40**
- 1.40

**SPECTRUM NO. 41**
- 1.41

**SPECTRUM NO. 42**
- 1.42

**SPECTRUM NO. 43**
- 1.43

**SPECTRUM NO. 44**
- 1.44

**SPECTRUM NO. 45**
- 1.45

**SPECTRUM NO. 46**
- 1.46

**SPECTRUM NO. 47**
- 1.47

**SPECTRUM NO. 48**
- 1.48

**SPECTRUM NO. 49**
- 1.49

**SPECTRUM NO. 50**
- 1.50

**SPECTRUM NO. 51**
- 1.51

**SPECTRUM NO. 52**
- 1.52

**SPECTRUM NO. 53**
- 1.53

**SPECTRUM NO. 54**
- 1.54

**SPECTRUM NO. 55**
- 1.55

**SPECTRUM NO. 56**
- 1.56

**SPECTRUM NO. 57**
- 1.57

**SPECTRUM NO. 58**
- 1.58

**SPECTRUM NO. 59**
- 1.59

**SPECTRUM NO. 60**
- 1.60

**SPECTRUM NO. 61**
- 1.61

**SPECTRUM NO. 62**
- 1.62

**SPECTRUM NO. 63**
- 1.63

**SPECTRUM NO. 64**
- 1.64

**SPECTRUM NO. 65**
- 1.65

**SPECTRUM NO. 66**
- 1.66

**SPECTRUM NO. 67**
- 1.67

**SPECTRUM NO. 68**
- 1.68

**SPECTRUM NO. 69**
- 1.69

**SPECTRUM NO. 70**
- 1.70

**SPECTRUM NO. 71**
- 1.71

**SPECTRUM NO. 72**
- 1.72

**SPECTRUM NO. 73**
- 1.73

**SPECTRUM NO. 74**
- 1.74

**SPECTRUM NO. 75**
- 1.75

**SPECTRUM NO. 76**
- 1.76

**SPECTRUM NO. 77**
- 1.77

**SPECTRUM NO. 78**
- 1.78

**SPECTRUM NO. 79**
- 1.79

**SPECTRUM NO. 80**
- 1.80

**SPECTRUM NO. 81**
- 1.81

**SPECTRUM NO. 82**
- 1.82

**SPECTRUM NO. 83**
- 1.83

**SPECTRUM NO. 84**
- 1.84

**SPECTRUM NO. 85**
- 1.85

**SPECTRUM NO. 86**
- 1.86

**SPECTRUM NO. 87**
- 1.87

**SPECTRUM NO. 88**
- 1.88

**SPECTRUM NO. 89**
- 1.89

**SPECTRUM NO. 90**
- 1.90

**SPECTRUM NO. 91**
- 1.91

**SPECTRUM NO. 92**
- 1.92

**SPECTRUM NO. 93**
- 1.93

**SPECTRUM NO. 94**
- 1.94

**SPECTRUM NO. 95**
- 1.95

**SPECTRUM NO. 96**
- 1.96

**SPECTRUM NO. 97**
- 1.97

**SPECTRUM NO. 98**
- 1.98

**SPECTRUM NO. 99**
- 1.99

**SPECTRUM NO. 100**
- 1.100
These different chromatographic agents would be expected to have specific reactions with each sort of group. Silver nitrate would be specific for chlorine groups while the formalin reagent reacts generally with benzene ring structures. Brom cresol green was used to identify acid compounds among the metabolites.

The following $r_f$ values were obtained for the experimental metabolites of methoxychlor. Table II gives the values obtained for the incubated samples of methoxychlor after thin layer chromatography and Table III gives the $r_f$ values of the theoretical metabolites and some suspected impurities that may be present in methoxychlor.

### Table II

<table>
<thead>
<tr>
<th>Experimental metabolites</th>
<th>$r_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>$r_f$</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>

Major metabolites only. Several weak spots were noticed and used for comparison also.

### Table III

<table>
<thead>
<tr>
<th>Theoretical metabolites</th>
<th>$r_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. methoxychlor</td>
<td>0.52</td>
</tr>
<tr>
<td>B. hydroxy derivative</td>
<td>0.00-0.30 streak</td>
</tr>
<tr>
<td>C. hydroxy ethylene</td>
<td>0.00-0.34 streak</td>
</tr>
<tr>
<td>D. hydroxy acid derivative</td>
<td>0.00-0.05 streak</td>
</tr>
<tr>
<td>E. ethylene derivative</td>
<td>0.32-0.60 streak</td>
</tr>
<tr>
<td>F. acid derivative of methoxychlor</td>
<td>0.21</td>
</tr>
<tr>
<td>diphenyl acetic acid</td>
<td>0.05-0.45 streak</td>
</tr>
<tr>
<td>1,1,1-tri-chloro-2,2-diphenyl ethane</td>
<td>0.77</td>
</tr>
</tbody>
</table>
The spots above were detected by two separate detection systems. The incubated sample was detected with the formalin reagent and the standards were detected with rhodamine B. The solvent system contained hexane:ether:acetic acid (120:35:5).

A second solvent was found that would permit separation of the hydroxy acid derivative (D). This system consisted of acetone:acetic acid (15:1). This system was planned for use with bile and liver samples from rats treated with methoxychlor. For this work animals were treated over a five day period with an oral dose of methoxychlor. They were then treated with an oral dose of methoxychlor prior to canulation of the bile duct. The bile was collected over a five hour period. The animal was then sacrificed and the liver removed. Both liver and bile samples were hydrolyzed with hydrochloric acid and extracted with ether. The ether extracts were spotted on the Eastman sheets and the strips placed in the acetone:acetic acid tank. Results of this study are shown below.
TABLE IV

<table>
<thead>
<tr>
<th>Hydrolyzed bile extract $r_f$</th>
<th>Hydrolyzed liver extract $r_f$</th>
<th>Hydrolyzed incubated sample $r_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.59</td>
<td>0.00-0.11 streak</td>
<td>0.82</td>
</tr>
<tr>
<td>0.70-0.85 streak</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE V

<table>
<thead>
<tr>
<th>standards $r_f$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bis-(hydroxy phenyl) acetic acid (D) 0.75</td>
</tr>
<tr>
<td>methoxychlor (A) 0.85</td>
</tr>
</tbody>
</table>

The small amount of data above indicates that methoxychlor is present in the incubated sample while there is a strong possibility that the hydroxy acetic acid (D) is present as a metabolite in bile from methoxychlor treated rats. In all the tests male rats were used.

Another system of comparison was necessary so vapor phase chromatography was utilized. Several tests were performed to determine the correct operating parameters of equipment. The tables below give the retention times obtained for the experimental and theoretical metabolites of methoxychlor.
The experimental metabolites were contained in the ether extracts of incubated samples of methoxychlor. Once again bile and liver from methoxychlor treated rats was extracted with ether and tested with vapor phase chromatography.

**TABLE VI**

Experimental metabolites from incubated methoxychlor. Retention times of major peaks.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>4.9</td>
</tr>
<tr>
<td>0.50</td>
<td>5.3</td>
</tr>
<tr>
<td>1.2</td>
<td>5.9</td>
</tr>
<tr>
<td>1.4</td>
<td>7.7</td>
</tr>
<tr>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>injector 285</td>
</tr>
<tr>
<td>3.4</td>
<td>column 225</td>
</tr>
<tr>
<td>4.5</td>
<td>detector 275</td>
</tr>
</tbody>
</table>

**TABLE VII**

Theoretical metabolites—retention time for major peaks.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>1.0</th>
<th>4.7</th>
<th>5.2</th>
<th>5.7</th>
<th>7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>diphenyl acetic acid</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methoxychlor (A)</td>
<td></td>
<td>4.7</td>
<td>5.2</td>
<td>5.7</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>ethylenic derivative of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methoxychlor (E)</td>
<td>4.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Temperatures: injector 285, column 225, detector 275
TABLE VIII

Retention times of experimental metabolites from an incubated sample of methoxychlor:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>0.80</td>
<td>2.20</td>
</tr>
<tr>
<td>1.2</td>
<td>3.4</td>
</tr>
<tr>
<td>1.6</td>
<td>Temperatures:</td>
</tr>
<tr>
<td>2.1</td>
<td>Injector 290</td>
</tr>
<tr>
<td>3.0</td>
<td>Column 225</td>
</tr>
<tr>
<td>3.2</td>
<td>Detector 290</td>
</tr>
<tr>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

TABLE IX

Theoretical metabolites—retention time for major peaks.

methoxychlor (A) 3.3 3.6 4.0 5.0
ethylenic derivative of methoxychlor (E) 3.4
hydroxy derivative (B) 9.3
hydroxy ethylene derivative (C) 8.2
bis-(p-anisyl) acetic acid (F) 6.0

Temperatures: injector 290, column 225, detector 290
Experimental metabolites from an incubated sample of methoxychlor and ether extracts of liver and bile from rats treated with methoxychlor. Retention times.

<table>
<thead>
<tr>
<th>Incubate</th>
<th>Liver</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Other peaks were neglected. A blank sample had no peak between 1.2 and 1.6.

Temperatures: injector 225, column 165, detector 250

The values in the table above are highly significant since bis(p-hydroxyphenyl) acetic acid (D) had the same retention time at the temperatures used in the trials above. Several repetitions were performed and the same peak was found in each trial. When compared to the $r_f$ values in tables IV and V, the hydroxy acid could be described as a metabolite of methoxychlor. However this information is still not strong enough for a complete confirmation of this metabolite. Using retention times and $r_f$ values though, the data does indicate that bis(p-hydroxyphenyl) acetic acid is a metabolite.
The results on the preceding pages seem to offer little encouragement for the experimental approach. This information could be misleading though due to several factors which were not thoroughly studied. Some confirmative data was obtained to show that bis (p-hydroxyphenyl) acetic acid (D) was among the metabolites from incubated methoxychlor. This was the only compound from the proposed chain that appeared in the experimental metabolites.

Reasons for this can be seen in the metabolic pathway determined for DDT. In this chain there are several intermediate metabolites which are only partially dehalogenated. It is quite possible that one or more of these partial breakdown products is the major component of methoxychlor metabolites.

The data shows that demethylation definitely occurs with methoxychlor. The actual percentage demethylated was difficult to determine since it varied with the different rats used in the studies. It did appear however that demethylation is a major pathway of methoxychlor detoxification in the male rat. The female rat may have a different system for this metabolism since demethylation did not occur rapidly in female tissue. It is possible that detoxification in the female occurs via hydroxylation of methoxychlor in one of its ring positions.
Much more research must be done to determine other metabolic chains for methoxychlor. The metabolites were detected by several chromatographic agents several of which would react only with the chlorine atoms on the metabolites. These tests indicated that at least two of the metabolites still contained chlorine atoms. These metabolites did not correspond to the ethylenic derivatives of methoxychlor in the theoretical scheme (see compounds C and E). It is possible that these chlorinated derivatives were produced by hydroxylation of the ring. However these compounds were not found in bile extracts of rats treated with methoxychlor. There was an indication that the bile did contain some of the hydroxy acid (D). Some of the possible intermediate metabolites that should be prepared and studied are shown below:

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{H} \\
& \quad \text{C} \\
& \quad \text{Cl} \\
\text{H} & \quad \text{C} \\
& \quad \text{Cl} \\
\end{align*}
\]

or

\[
\begin{align*}
\text{CH}_2\text{O} & \quad \text{H} \\
& \quad \text{C} \\
& \quad \text{Cl} \\
\text{H} & \quad \text{C} \\
& \quad \text{Cl} \\
\end{align*}
\]

or

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{H} \\
& \quad \text{C} \\
& \quad \text{Cl} \\
\text{H} & \quad \text{C} \\
& \quad \text{Cl} \\
\end{align*}
\]
The above six compounds are all possible intermediates in the proposed methoxychlor chain of metabolism. Preparation of the above compounds could be rather difficult but not impossible. Compound (I) could probably be made by condensing equal amounts of phenol and anisole with chloral hydrate and then refluxing with alcoholic potassium hydroxide. Compound (J) could be made by condensing an equimolar mixture of phenol and anisole with glyoxylic acid. No attempt was made to synthesize these compounds although a later effort may be made.

Possible metabolites due to ring hydroxylation are shown below:

Presently no possible theoretical synthesis could be proposed due to the complexity of the products.
As one can see, the number of metabolites which can come from methoxychlor is rather large. Several of these would only be expected in minute amounts, but they could exist. Several more problems must be solved before the project can be considered complete. Initially we had hoped to make a kinetic study on methoxychlor. It is first necessary to identify the major metabolites from the system. Our initial kinetic approach would have been to measure the amount of free chlorine in the incubate at varying times. Time did not allow us to make this determination though. Furthermore, no attempt was made to explain kinetic data in terms of demethylation rates.

Concluding we can say that the problem has been attacked, but results only indicate that we have opened the door to further research on methoxychlor metabolism.
BIBLIOGRAPHY


6. White, W. and Sweeney, T.; "The Metabolism of 2,2 bis(p-chlorophenyl) 1,1,1 trichlorethane (DDT)", Public Health Reports 60, 66 (1945).


