Non-enzymatic Conversion of Dipterex into DDVP and Their Inhibitory Action on Enzymes. : Studies on the Mode of Action of Dipterex I

Author(s)
MIYAMOTO, Junshi

Citation
防虫科学 24(3): 130-137

Issue Date
1959-08-31

URL
http://hdl.handle.net/2433/158139

Type
Departmental Bulletin Paper

Textversion
publisher

Kyoto University
Résumé

The degradation of Malathion emulsifiable concentrate during storage comes up to 20 to 30% in a year; this is a serious matter for the manufacture and application of the concentrate.

In this report is given an outline of the study since 1955 upon the stability of Malathion e. c.

(1) The degradation of Malathion e. c. depends mostly upon the kind of the emulsifiers and organic solvents, the moisture in the formulation, the storage temperature etc.

(2) Xylene and benzene gave good results as solvent.

(3) Moisture in the formulation is mostly derived from emulsifiers and has a very important effect upon the degradation of Malathion. pH of the emulsifiers (×100) is also important; optimum pH is 7.5 to 8.0 (Table 9).

(4) Polyoxyethylene glycol (PEG) in the emulsifiers affects the degradation of Malathion (Table 3, Table 5, X, XI, XII, XIII).

(5) Use of the molecular-distilled emulsifier, which contains very little quantity of PEG, brings forth low degradation. (Table 2, Table 5).

(6) It is recommendable that NaOH etc., which are used as catalysts, are not be neutralized in the process of synthesizing the emulsifiers for use in Malathion e. c., i.e., it is not necessary to adjust pH at 6 to 7; pH = 7.5 to 8.0 is quite all right. Namely, the emulsifier No.7 (Table 2) gave lower degradation of Malathion than the other.

(7) More than 4.0 is desirable for the pH of Malathion e. c. (×100).

(8) The essentials for preventing the degradation are written at above (1)－(7). It may be, however, preventative measures to use smaller quantity of emulsifiers, which have most important relations with the degradation of Malathion e. c.
Dipterex was proved to undergo dehydrochlorination and molecular rearrangement to form DDVP not only under neutral but even under slightly acidic conditions. By inhibitory experiment of Dipterex and DDVP on acetylcholinesterase and chymotrypsin at various pHs, it was revealed that Dipterex itself shows little or no inhibitory action. Inhibition caused by Dipterex, therefore, is attributed to DDVP derived from Dipterex. 

The insecticidal action of Dipterex in vivo should be also due to DDVP which is expected to form spontaneously in the insect or plant body under physiological conditions.

Introduction

Dipterex or O,O-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate has attracted peculiar attention of biochemists as well as entomologists since discovery of its excellent toxic effects to the house fly, Musca domestica L., and of its weaker influence to mammals than other phosphorus containing chemicals. The author has observed recently that this compound undergoes dehydrochlorination and molecular rearrangement successively to form DDVP or O,O-dimethyl-dichlorovinyl phosphate in the neutral or even in the acidic media, similarly as under the alkaline condition1-4. 

This paper concerns with experimental results about spontaneous change of Dipterex and the inhibitory action of Dipterex and DDVP formed on acetylcholinesterase of the house-fly heads and also on chymotrypsin.

Experimental

Dipterex was synthesized from dimethyl hydrogen phosphite and chloral5, and recrystallized from CCl4 and then from ether. Its melting point and the results of elementary analysis are shown below:

<table>
<thead>
<tr>
<th>Property</th>
<th>Measured</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mp.</td>
<td>78.5-79.5°C</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>18.34%</td>
<td>18.65%</td>
</tr>
<tr>
<td>H</td>
<td>3.27%</td>
<td>3.10%</td>
</tr>
<tr>
<td>Cl</td>
<td>40.26%</td>
<td>41.50%</td>
</tr>
</tbody>
</table>

Purity of this sample was proved to be 98.5% by polarographic method6. DDVP was prepared by dehydrochlorination of the Dipterex7. Its physical properties and the results of elementary analysis are shown below:

<table>
<thead>
<tr>
<th>Property</th>
<th>Measured</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>nD</td>
<td>1.4590</td>
<td></td>
</tr>
<tr>
<td>d21</td>
<td>1.414</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>22.61%</td>
<td>21.70%</td>
</tr>
<tr>
<td>H</td>
<td>3.82%</td>
<td>3.16%</td>
</tr>
<tr>
<td>P</td>
<td>14.48%</td>
<td>14.00%</td>
</tr>
<tr>
<td>Cl</td>
<td>30.73%</td>
<td>32.20%</td>
</tr>
</tbody>
</table>

Purity of both compounds were also examined by infrared absorption spectra. Acetylcholine perchlorate was prepared from commercial acetylcholine chloride8, and white needle crystal of mp. 114-116°C was obtained. White crystal of benzoylcholine of mp. 241-243°C was prepared as iodide by the method described in Organic Synthesis9. L-Phenylalanine ethylester hydrochloride was prepared by the esterification of commercial L-phenylalanine in absolute ethanol9 and recrystallized from absolute ethanol. Its melting point showed 147.5-149°C.

Enzyme preparation, assay of activity, and inhibition experiment. Chymotrypsin used was crystalline powder from Nutritional Biochemical Corp. Its activity was assayed at pH 6.5, 30°C according to the method of Parks and Plaut10, L-phenylalanine ethylester hydrochloride being used as substrate. Inhibition experiments by Dipterex and DDVP were performed as follows: chymotrypsin and inhibitor were preincubated in 0.01M phosphate buffer at definite pH, at 4°C for 24 hrs. After pH was readjusted to 6.5, enzyme activity was determined. Initial velocity observed was compared with that of the control, and inhibitory effect caused was determined. In the case of acetylcholinesterase, inhibitory action of Dipterex and DDVP in vitro were investigated manometrically by using supernatant from the homogenate of the adult house-fly heads by centrifugation at 6,000 rpm for 7 mins. Before enzyme solutions were added, inhibitors
Results and Discussion

1. Inhibition of chymotrypsin by Dipterex and DDVP

As shown in Fig. 1, DDVP was observed to inhibit chymotrypsin to nearly the same extent at pH 6.5 and at pH 7.6, while inhibition by Dipterex was found to depend remarkably on pH of preincubation. Concentration of Dipterex and DDVP required to cause 50% inhibition are shown in Table 1.

![Graph showing inhibition of chymotrypsin by Dipterex and DDVP](image)

**Table 1.** IN₅₀ of Dipterex and DDVP on chymotrypsin

<table>
<thead>
<tr>
<th>pH during preincubation</th>
<th>IN₅₀ (M)</th>
<th>Dipterex</th>
<th>DDVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>&gt;1×10⁻¹</td>
<td>4.2×10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>4.5×10⁻³</td>
<td>1.5×10⁻⁴</td>
<td></td>
</tr>
</tbody>
</table>

(In₅₀) under these experimental conditions were given in Table 1.

When Dipterex and chymotrypsin were preincubated at pH 5.0, 4°C for 24 hrs., no inhibition appeared, while under the same condition DDVP inhibited the action of enzyme to a certain extent; 5×10⁻³M and 1.25×10⁻³M of DDVP caused 37% and 5% inhibition respectively when preincubated with the enzyme at pH 5.0, 6°C for 20 hrs.. As to the difference in inhibitory potency between these inhibitors, it might be considered as follows.

Phosphate bond P-O-C in DDVP molecule is presumed to be relatively stable with the change of pH, while phosphonate bond P-C in Dipterex molecule is very sensitive to pH, and Dipterex is presumed to be inhibitory only when it is converted into DDVP by preincubation.

The higher pH solutions may cause the more conversion of Dipterex and makes it the more effective.

![Chemical structures of Dipterex and DDVP](image)

2. Formation of DDVP from Dipterex under neutral and acidic conditions

The above presumption was tried to demonstrate by the following experiments. Liberation of hydrochloric acid concurrent with this transformation was ascertained by CO₂ evolution from CO₂-bicarbonate buffer. The results were shown in Fig. 2. Thus formation of acid from Dipterex in the neutral medium was demonstrated.
Fig 2. CO₂ evolution from CO₃bicarbonate buffer at various pHs by dehydrochlorination of Dipterex at 37°C. 2×10⁻²M of Dipterex was used. Reaction mixture, total volume 3 ml.

Even at pH 5.3 this reaction was observed to proceed very slowly (11 µl /3 ml reaction mixture /30 mins. when 6×10⁻²M Dipterex was used). This acid production was proved to be neither accelerated by the addition of chemicals which were used in the inhibition studies of chymotrypsin and acetylcholinesterase, nor stimulated by fly head preparation. It seems to be evident, therefore, that the acid formation from Dipterex depends only on the pH. Formation of DDVP after liberation of hydrochloric acid was also identified by infrared absorption spectra of chloroform extract of the reaction mixture.

The results obtained were shown in Fig. 3. The absorption band at 6.07 μ designates the presence of terminal C=C bond, and the band at 7.84 μ coincides with that of P=O bond in phosphate.

The further proof for the decomposition of Dipterex to DDVP was given by the following experiment. Dipterex was dissolved in 0.1M phosphate buffer of various pHs and vapor toxicity was investigated. The aqueous solution of Dipterex was fairly acidic and showed no vapor toxicity at all. Even at pH 5.0 vapor toxicity was not observed yet, while at pH 5.5 the toxicity appeared, and with the rising of pH, it was seen to become stronger. At pH 7.6 the toxicity was nearly same as that of DDVP (Table 2).

Wave length μ

Fig 3. Infrared absorption spectra of the chloroform extract of Dipterex solution after standing at 37°C. A. 40 ml of 10⁻²M Dipterex solution at pH 7.6, (1) for 30 min, (2) 90 min. B. 40 ml of 3×10⁻²M Dipterex solution at pH 6.5 (1) for 80 min, (2) 160 min., and extracted with 4 ml of CHCl₃. This CHCl₃ layer was dehydrated with anhydrous Na₂SO₄.

(3) in B; DDVP control (5×10⁻²M of DDVP in CHCl₃)

Table 2. Vapor toxicity of Dipterex and DDVP

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Kill %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
</tr>
<tr>
<td>Dipterex (in H₂O)**</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>DDVP ** ** ** **</td>
<td>100</td>
</tr>
</tbody>
</table>

* means of three replicates.  ** pH of the aqueous solution; 1% pH 3.3, 0.2% pH 4.3.
* * * diluted with the same buffer solution.  ** ** ** dissolved in small amount of propylene glycol and diluted with H₂O.

133
The rate at which Dipterex converts into DDVP was determined at pH 6.5 and at pH 7.6 with the above method. The results obtained were shown in Fig. 4 and Fig. 5. Acid formation (DDVP formation) proceeded proportionally with the time during the period when decrease of Dipterex was not so much. Thus the process of DDVP formation may be represented by the first-order reaction \( C = C_0(1 - e^{-kt}) \). The initial first-order rate constant \( K \) was determined as shown in Table 3. DDVP formation was 9 times slower at pH 6.5 than at pH 7.6. From these results in company with those in Fig. 2 and in Table 2, this reaction seems to fall with the lowering of the pH of the medium, and at last cease at pH 5.0.

![Figure 4. Acid formation from Dipterex at pH 6.5, 37°C. Number on each curve; concentration of Dipterex used (M)](image)

![Figure 5. Acid formation from Dipterex at pH 7.6, 37°C. Number on each curve; concentration of Dipterex used (M)](image)

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial concentration of Dipterex (( C_0 )) M</th>
<th>Acid formation during 10 mins. (( CKt )) M</th>
<th>Rate constant (K) min(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>( 4.0 \times 10^{-2} )</td>
<td>( 0.265 \times 10^{-3} )</td>
<td>( 6.6 \times 10^{-4} )</td>
</tr>
<tr>
<td></td>
<td>( 2.0 )</td>
<td>( 0.140 )</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>( 1.0 )</td>
<td>( 0.085 )</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>( 0.5 )</td>
<td>( 0.035 )</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>mean ( 7.3 \times 10^{-4} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>( 3.0 \times 10^{-2} )</td>
<td>( 1.80 \times 10^{-3} )</td>
<td>( 6.0 \times 10^{-3} )</td>
</tr>
<tr>
<td></td>
<td>( 1.0 )</td>
<td>( 0.78 )</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>( 0.4 )</td>
<td>( 0.26 )</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>( 0.2 )</td>
<td>( 0.135 )</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>mean ( 6.7 \times 10^{-3} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Inhibition of fly head acetylcholinesterase by Dipterex, DDVP

Enzyme preparation from the adult house-fly head contains so-called acetylcholinesterase as it hydrolyzes acetylcholine well and is inhibited by acetylcholine at high concentration and it shows little activity on to benzoylcholine (Fig. 6). Inhibition experiments by Dipterex and DDVP was carried out with this preparation. The results obtained were given in Fig. 7, 8 and Table 4. Preincubation at pH 7.6 and pH 6.5 makes Dipterex inhibitory to the markedly different extent, while preincubation of DDVP at the same pHs any difference was scarcely observed between these two pHs.

DDVP considered to be formed from Dipterex during preincubation were calculated and shown in column 3 in Table 4. Its amount agreed well with that of DDVP necessary to cause 50% inhibition (last column in Table 4). The feeble inhibition observed by Dipterex without preincubation may be attributed also to the conversion of Dipterex in the buffer solution during the time for assay of enzyme activity.

The similar inhibition was also apparent in the experiment at pH 5.3 where DDVP
Fig 6. Substrate specificity of adult house fly head preparation. 40 heads/ml (containing 25 μM reduced glutathione, 300 μM NaCl, 89 μM MgCl₂) were homogenized and centrifuged at 6,000 rpm for 7 mins. 0.3 ml of supernatant was used. Reaction mixture (total volume 3 ml) contains 450 μM NaCl, 120 μM MgCl₂, 7.5 μM reduced glutathione, 72 μM NaHCO₃, enzyme and substrate. Gas phase 5% CO₂, 95% N₂. pH 7.6, 37°C. A.Ch.; acetylcholine. Bz.Ch.; benzoylcholine.

Fig 7. Inhibition of fly head acetylcholinesterase by Dipterex, DDVP at pH 6.5, 37°C after preincubation of inhibitors in the buffer solution for various time length.

Reaction mixture, same as in Fig 6 except that inhibitor added and 15 μM acetylcholine, 126 μM NaHCO₃ used. Gas phase 100% CO₂. Preincubation of inhibitor in the buffer for 0(□—□), 5(■—■) and 20 mins. (△—△).

formation from Dipterex occurs extremely slowly. At this pH it needs 10⁻⁴ M of DDVP to cause 50% inhibition, while IN₅₀ for Dipterex is 2×10⁻⁵ M when without preincubation. From these facts, Dipterex is determined to possess no inhibitory potency by itself.

It has been assumed that when Dipterex is used as an insecticide, conversion of Dipterex into DDVP in vivo may be due to the action of a specific enzyme "dehydrochlorinase" present in the insect or plant body, but this presumption has not yet been demonstrated by experimental

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Dipterex M</th>
<th>DDVP formed from Dipterex M*</th>
<th>DDVP M</th>
<th>Dipterex/DDVP***</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5</td>
<td>t=0*</td>
<td>&gt;4.0×10⁻³</td>
<td>—</td>
<td>1.4×10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.7×10⁻⁴</td>
<td>1.4×10⁻⁶</td>
<td>1.2×10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.0×10⁻⁵</td>
<td>1.3×10⁻⁶</td>
<td>1.3×10⁻⁶</td>
</tr>
<tr>
<td>pH 7.6</td>
<td>t=0</td>
<td>4.3×10⁻⁴</td>
<td>—</td>
<td>1.2×10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.1×10⁻⁵</td>
<td>1.0×10⁻⁶</td>
<td>1.1×10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.1×10⁻⁶</td>
<td>1.2×10⁻⁶</td>
<td>1.2×10⁻⁶</td>
</tr>
</tbody>
</table>

* time length (min) of preincubation of inhibitors in the buffer solution before addition of enzyme. ** DDVP considered to form from IN₅₀ of Dipterex under each experimental condition (calculated using rate constant K(pH7.6)=6.7×10⁻⁵ min⁻¹, K(pH6.5)=7.3×10⁻⁴ min⁻¹). *** ratio of potency (IN₅₀ of DDVP calculated/found).
1. Spontaneous conversion of Dipterex into DDVP was ascertained under neutral and acidic pH conditions. This conversion seems not to occur practically below pH 5.0, and with the rising of pH the rate increases proportionally.

2. Inhibition of Dipterex and DDVP on fly head acetylcholinesterase and on chymotrypsin was investigated, and it became clear that the apparent inhibitory action of Dipterex in vitro is due to the action of DDVP formed by conversion and Dipterex itself is not inhibitory.

3. The relation of this spontaneous conversion of Dipterex into DDVP to the insecticidal action of the former and to its lower mammalian toxicity was also discussed.

Summary

The results will be presented in the continuing papers.

1. Spontaneous conversion of Dipterex into DDVP was ascertained under neutral and acidic pH conditions. This conversion seems not to occur practically below pH 5.0, and with the rising of pH the rate increases proportionally.

2. Inhibition of Dipterex and DDVP on fly head acetylcholinesterase and on chymotrypsin was investigated, and it became clear that the apparent inhibitory action of Dipterex in vitro is due to the action of DDVP formed by conversion and Dipterex itself is not inhibitory.

3. The relation of this spontaneous conversion of Dipterex into DDVP to the insecticidal action of the former and to its lower mammalian toxicity was also discussed.

Acknowledgement

The author is indebted to Mr. K. Iwai, Mr. K. Sugiyama, Mr. Y. Okuno, and Dr. N. Oi in this laboratory for their technical assistance in his work.

References


27. ニカメイガの卵に寄生しているズイムシアカタマワタチに及ぼす農薬の影響 農薬の
寄生に及ぼす影響について 第1報 習熟羽・桑名貞夫（養原農薬研究所・昆虫研究室）34. 7. 28 受理
ニカメイガの卵とそれに寄生しているズイムシアカタマワタチに対する7種類の農薬の影響を実験
法による室内実験によって調べた。一般にニカメイガ卵に対する作用の強い薬剤はズイムシアカ
タマワタチに対する影響を強く作用したが、エンドリン、リンデン、ダイアソノン乳剤を含むズイム
シアカタマワタチに強く作用し、PMA乳剤はニカメイガ卵に強く作用するという結果を得た。

殺虫剤の散布が目的とする害虫のみならず、その天
敵の影響を考慮したことに、この実験群が各種の生物の殺虫剤に影響する一因となり、害虫の異常発生を生じ、作物の被害を
増加することが知られている1)2)。水田における農薬
の使用がニカメイガ卵に寄生しているズイムシアカ
タマワタチにどのような影響を及ぼしているかを知る
手がかりとして、室内実験によって両者の対照した
様々な農薬の殺虫力を比較検討した。

結果を報告するに先だって卵卵単位および毎卵数を観察
えた京都大学農学部昆虫学研究室、内田俊男教授、高橋
史雄氏、ならびに実験農薬研究所昆虫研究所、坪井武
夫、篤原覚の両氏の皆様に深謝の謝意を表する。

実験材料および方法

1959年6月15日静岡県清水市清水近くの栽培でニカメイガの卵卵を多数採集し、ズイムシアカタマワタチ
が寄生していると思われる卵類の卵を基準に
して、これらを黒黒変色卵、黒灰色変色卵、灰色卵、白
色卵の4段階に区分けし、実験に供した。4段階に区
分けしたニカメイガ卵の卵卵の発生の程度をその卵
化曲線より推定したところ、黒黒変色卵は孵化後2.1
日、黒灰色卵は3.8日、灰色卵は4.5日、白色卵は
6.2日となった。またズイムシアカタマワタチの発育
程度についてもニカメイガ卵を用いた同じ構築
法により推定することが出来る。すなわち黒黒変色卵は
羽化前3.3日、黒灰色卵は5.0日、灰色卵は7.4日、


Addendum

Just when the manuscript was almost completed, a paper by R. L. Metcalf et al. was published under the title of “Toxic action of Dipterex and DDVP to the house fly” (J. Econ. Ent. 52, 44 1959), where they reported almost the same results as the author obtained which support spontaneous conversion of Dipterex into DDVP under similar conditions.