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<th>Enzymic Dehydrochlorination of Trichlorfon by the Digestive Juice of the Silkworm, Bombyx mori L</th>
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<td>Author(s)</td>
<td>SUGIYAMA, Hiroshi; SHIGEMATSU, Hajime</td>
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The toxicity of trichlorofon (O,O-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) is higher in the oral application than in the topical application to the silkworm larva. In the preceding paper it was recovered that trichlorofon was rapidly converted into DDVP (O,O-dimethyl 2,2-dichlorovinylphosphate), which is a powerful inhibitor of cholinesterase, in the digestive juice through dehydrochlorination, but not in the hemolymph of the silkworm larva in vitro. It has been suggested that the insecticide was rapidly dehydrochlorinated in a weak alkaline solution. We found the occurrence of active conversion of trichlorofon even with use of the neutral digestive juice. Therefore, we will pay our attentions to a problem if the dehydrochlorination of trichlorofon is dependent on an enzymic reaction or simply on the alkalinity of the digestive juice.

**Materials and Methods**

The digestive juice from the larvae of the silkworm, *Bombyx mori*, of a hybrid between the strains Nichi 124 and Shi 124 was collected on the fourth to sixth days of the fifth instar, after one day of fasting, with vomiting by an electric stimulation. The juice was stored at -20°C until use. After thawing, the supernatant fluid resulted after the centrifugation of the juice for 10 minutes at 1,000 G was subjected to the assay.

Trichlorofon was purified from 80% soluble powdery formulation by recrystallization from *n*-hexane. A technical DDVP of 98% purity was dissolved in ether and washed with distilled water for several times. When the solvent was removed under a diminished pressure, DDVP was obtained as a viscous liquid and stored in an ampule until use.

Experiments on the non-enzymic conversion of trichlorofon into DDVP were carried out with a mixture of 0.8 ml of a definite pH in 0.5M Tris (hydroxyethylaminomethane)-NaOH-HCl buffer solution and 2.57 mg trichlorofon in 0.2 ml of distilled water at 28°C. The reaction products were once extracted from the reaction mixture with 4 ml of acetoniitrite. Before the determination with spectrophotometer, acetoniitrite extracts were cleaned by the centrifugation.

Experiments with the digestive juice of larva were carried out by mixing 0.2 ml of the juice, 0.5 ml of Tris-HCl buffer, 0.1 ml of 10^4*M KCN solution and 2.57 mg trichlorofon in 0.2 ml and incubated at 35°C. The reaction products were extracted with acetoniitrite as described above. For an anaerobic reaction, a Thunberg tube was used after sucking air for 3 minutes by an aspirator.

Acetonitrile extracts were optically examined with ultraviolet absorptions. Careful attention should be paid to shifting of absorption maximum with different kind of solvents or amounts of the digestive juice added. So far we examined, DDVP...
in acetonitrile solution showed an absorption maximum at 212~214 m\(\mu\), while in acetonitrile including Tris-NaOH-HCl buffer, the absorption maximum of DDVP shifted to 218 m\(\mu\). Moreover, in the solution added with the digestive juice, the absorption maximum shifted to 220~230 m\(\mu\). Silica gel thin-layer chromatography, developing by benzene-methanol (9:1, v/v), was employed to separate decomposition products of trichlorofon. Visible spots of chromatogram under ultraviolet light were extracted with acetonitrile and assayed for ultraviolet and infrared absorptions. By this solvent system, RF values for trichlorofon, DDVP, and another decomposition product were 0.0~0.10, 0.55~0.80, and 0.20~0.35, respectively.

**Results**

*Determination of DDVP by Ultraviolet Absorption:* The C=C double bond of DDVP is responsible to the absorption of ultraviolet light. Possibility of the determination of DDVP was examined by the ultraviolet absorption in acetonitrile. Various amount of DDVP dissolved in 0.5 ml of acetonitrile and 1 ml of 0.5 M Tris-HCl buffer (pH 7.0) was vigorously shaken with 4 ml of acetonitrile and then centrifuged for 5 minutes at 1,000 G. Acetonitrile layer was assayed for optical absorption at 218 m\(\mu\). The optical absorption of DDVP was linearly related to the concentration of the compound (A curve in Fig. 1). Recovery of DDVP from the digestive juice was tested as follows: various amounts of DDVP dissolved in 0.5 ml of acetonitrile were mixed with 0.2 ml of the juice and 0.8 ml of the buffer (pH 7.0), and DDVP was extracted with 4 ml of acetonitrile by vigorous shaking as mentioned above. Since DDVP in the acetonitrile extract showed absorption maximum at 230 m\(\mu\), the optical density at 230 m\(\mu\) was plotted against the concentration of DDVP initially added. Results were shown with curve B in Fig. 1. Recovery of DDVP was 64~67% in this case. Shift of the absorption maximum and change of recovery of DDVP had to be pre-examined in experiments with use of the digestive juice. As previously mentioned, trichlorofon was converted into at least two compounds, detectable on the thin-layer chromatogram, which seem to be DDVP and its analogue, and the latter compound showed also ultraviolet absorption as described later. Therefore we had to temporarily express the amount of conversion products of trichlorofon as DDVP equivalence calculated from the lines of Fig. 1. Trichlorofon in acetonitrile did not absorb the ultraviolet light at the range between 200 and 260 m\(\mu\) as reported by Tokumitsu.

![Fig. 1. Spectrophotometric determination of DDVP. A: in the absence of the digestive juice, measured at 218 m\(\mu\). B: in the presence of the digestive juice, measured at 230 m\(\mu\).](image)

*Non-enzymic Conversion of Trichlorofon into DDVP:* Trichlorofon was rapidly converted into DDVP in an alkaline solution. The time course of DDVP formation from trichlorofon under various pH was shown in Fig. 2. The higher in pH value of the solution, the higher in the initial velocity of the conversion of trichlorofon. However, trichlorofon in an alkaline solution was not completely degraded. For instance, DDVP produced at pH 9.8 in 60 minutes-reaction amounted to 1.05 mg, only about half of trichlorofon initially added, and then slightly fell down in accordance to reaction time; it was not clear whether ODVP was further converted into other products or not. At pH 9.0 it took almost 100 minutes to arrive at the same maximum level of DDVP production at pH 9.8. In the reactions at pH 8.1 and 7.1, the reaction velocity decreased remarkably.

*Conversion of Trichlorofon into DDVP in the Digestive Juice in Vitro:* When the digestive juice was left to stand after centrifugation, pH was reduced from about 10 to 7 within 30 minutes. Since it was difficult to keep constant the pH of
Table 1. Activity of the digestive juice on dehydrochlorination of Trichlorofon under aerobic and anaerobic conditions at pH 7.0. The reaction mixture was prepared according to the methods and incubation was allowed to proceed for 30 minutes at 35°C.

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Amount of DDVP formed (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aerobic</td>
<td>60</td>
</tr>
<tr>
<td>anaerobic</td>
<td>130</td>
</tr>
<tr>
<td>aerobic + KCN (1x10^-3M)</td>
<td>225</td>
</tr>
</tbody>
</table>

Table 2. Effect of KCN addition to the digestive juice on an aerobic reaction at pH 7.0. Reaction condition was the same as Table 1.

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Amount of DDVP formed (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCN added, 1x10^-4M</td>
<td>195</td>
</tr>
<tr>
<td>KCN added, 1x10^-3M</td>
<td>475</td>
</tr>
<tr>
<td>KCN added, 1x10^-2M</td>
<td>40</td>
</tr>
<tr>
<td>KCN not added</td>
<td>trace</td>
</tr>
<tr>
<td>Digestive juice omitted (no KCN)</td>
<td>15</td>
</tr>
<tr>
<td>With boiled digestive juice (no KCN)</td>
<td>trace</td>
</tr>
</tbody>
</table>

Fig. 2. The amount of DDVP converted non-enzymically from 2.57 mg of trichlorofon in various pH solutions.

the digestive juice with a dilute buffer, 0.5 M Tris-HCl buffer was employed in this investigation. As shown in Table 1, activity was not significantly detected in the reaction under aerobic condition, but clearly observed under anaerobic condition or by an addition of KCN. Optimum concentration of KCN was found to be around 1x10^-3M (Table 2). Reduced glutathion or NADPH2 did not accelerate the activity. Under the aerobic condition of the digestive juice, the conversion
was shifted to longer wave length with use of larger volume of the digestive juice (Fig. 5).

The conversion of trichlorofon into DDVP in the digestive juice was examined under various pH values ranging from 5 to 11 (Table 3). Tris-HCl buffer was employed, but over pH 11 and below 7, NaOH and HCl were added to give appropriate values, respectively. The reaction without the digestive juice was set as a reference. It was noticed at first from the results in Table 3 that the values of optical absorption at zero time reactions were not ignorant, depending upon pH values, and were supposed to be formed during the period of extraction of the reaction products. These values responded to the non-enzymic reaction shown in Fig. 2.

A remarkable production of DDVP in these experiments was observed at pH 7 to 8. Amounts of DDVP formed by non-enzymic conversion of trichlorofon were observed to be 230 and 65 µg at pH 8.3 and 7.0, respectively, while in enzymic reaction 555 and 425 µg of DDVP were formed at pH 8.2 and 6.3, respectively. Larger amount of produced DDVP in enzymic reaction at a high pH range, for example, 780 µg at pH 9.0, might be resulted by an additional effect of non-enzymic and enzymic conversions. However, the enzymic action related to the dehydrochlorination of trichlorofon in the digestive juice was not effective above pH 10. This enzyme seemed to have the optimum pH at neutral or rather slightly acidic.

Attempts for Purification of the Enzyme: Preliminary trials for purification of the enzyme were carried out. This enzyme could be fractionated by columns of either Sephadex G-50 or G-100. Using the column of DEAE-cellulose equilibrated by 0.05M Tris buffer (pH 7.4), fractionated by NaCl solution with the linear gradient of its concentration from 0 to 1.0M, the active fractions were eluted with around 0.6M salt concentration, after major peaks of juice protein, as presented in Fig. 6.

Zymography was attempted to visualize the active band of protein on paper strip through electrophoresis. A small amount of the digestive juice or the active eluate from the column was placed on strips of Toyo filter paper no. 51A and the electrophoresis was performed for 3 hours.
Table 3. Conversion of trichlorofon into DDVP in the digestive juice at various pH.

The reaction mixture was prepared according to the methods. Incubation
the was allowed to proceed for 15 minutes at 35°C.

<table>
<thead>
<tr>
<th>pH of the reaction mixture</th>
<th>Optical density at zero time</th>
<th>Increase of optical density at 15 min.</th>
<th>Amount of DDVP formed in 15 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Reactions without the digestive juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.4</td>
<td>1.09 (223mÇ)</td>
<td>0.420 (227mÇ)</td>
<td>335Çg</td>
</tr>
<tr>
<td>10.0</td>
<td>0.77 (222mÇ)</td>
<td>0.620 (225mÇ)</td>
<td>495</td>
</tr>
<tr>
<td>9.0</td>
<td>0.396 (221mÇ)</td>
<td>0.614 (223mÇ)</td>
<td>490</td>
</tr>
<tr>
<td>8.3</td>
<td>0.306 (219mÇ)</td>
<td>0.292 (220mÇ)</td>
<td>232</td>
</tr>
<tr>
<td>7.5</td>
<td>0.243 (218mÇ)</td>
<td>0.095 (217mÇ)</td>
<td>75</td>
</tr>
<tr>
<td>7.0</td>
<td>0.218 (218mÇ)</td>
<td>0.087 (217mÇ)</td>
<td>65.5</td>
</tr>
<tr>
<td>(B) Reactions with the digestive juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>1.08 (228mÇ)</td>
<td>0.223 (234mÇ)</td>
<td>280Çg</td>
</tr>
<tr>
<td>9.9</td>
<td>1.08 (230mÇ)</td>
<td>0.233 (235mÇ)</td>
<td>290</td>
</tr>
<tr>
<td>9.0</td>
<td>0.522 (226mÇ)</td>
<td>0.653 (230mÇ)</td>
<td>780</td>
</tr>
<tr>
<td>8.2</td>
<td>0.288 (224mÇ)</td>
<td>0.462 (227mÇ)</td>
<td>555</td>
</tr>
<tr>
<td>7.2</td>
<td>0.186 (222mÇ)</td>
<td>0.349 (223mÇ)</td>
<td>425</td>
</tr>
<tr>
<td>6.3</td>
<td>0.223 (221mÇ)</td>
<td>0.350 (223mÇ)</td>
<td>425</td>
</tr>
<tr>
<td>5.4</td>
<td>0.136 (223mÇ)</td>
<td>0.308 (221mÇ)</td>
<td>375</td>
</tr>
<tr>
<td>(C) Rough estimation of DDVP formation owed to enzymic action of the digestive juice (B-A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td></td>
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<td></td>
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<tr>
<td>9.0</td>
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<tr>
<td>8.3</td>
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<tr>
<td>7.1</td>
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<tr>
<td>6.3</td>
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<td></td>
</tr>
<tr>
<td>5.4</td>
<td></td>
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</tbody>
</table>

a Wave-length of absorption maximum was shown in parentheses
b Enzymic conversion seems not to be accounted
c Amount of non-enzymic conversion was extrapolated from data of (A)

with 1.0 mA/cm in the cold room, using Veronal buffer (pH 8.6). After the run, the paper strip was gently sprayed by 0.1M Tris buffer, pH 6.6, and coated by trichlorofen solution, followed by incubation for 30 minutes at 35°C. And then, the paper was dried and colored by the adequate methods for DDVP or protein. The results suggested that the active band had the highest mobility, aparted from major bands of juice protein.

Another Derivative of DDVP: Another derivative of DDVP suggested in previous experiments was separated by thin-layer chromatography on silica gel plate of 500 µ thickness. The substance showed Rf value 0.20-0.35 with the benzene-methanol solvent system and was extracted from the plates by acetonitrile, and was assayed by ultraviolet and infrared spectrophotometry and chemical reaction. As shown in Fig. 7, a large absorption at 212 mµ which was characteristic for DDVP was recognized in ultraviolet absorption spectrum of this compound. Infrared spectra of DDVP and of this compound were shown in Fig. 8. As compared with the spectrum of DDVP, this compound showed the following natures: OH vibration occurred near 3,200 cm⁻¹; two new bands absorption at 1, 122 and 1, 075 cm⁻¹ appeared instead of 1,150 and 1,050 cm⁻¹; a characteristic absorption of DDVP at 980 cm⁻¹ was not found, but new absorption bands were found at 967 and
Fig. 6. Elution pattern from DEAE-cellulose column of an enzyme catalyzing dehydrochlorination of trichlorofon from the digestive juice. A linear NaCl gradient (0 to 1.0 M) was employed and fractions of 3 ml were collected. -: protein, ---: enzyme activity, ---: NaCl concentration.

Fig. 7. Ultraviolet absorption spectra of DDVP and of another derivative separated by thin-layer chromatography. I: extract from Rf 0.55~0.80 region (DDVP), II: extract from Rf 0.20~0.35 region; chromatoplate, silica gel, 500μ in thickness; developing solvent system, benzene-methanol (9: 1, v/v).

943 cm⁻¹ suggesting the presence of the vinyl moiety; in the 900-700 cm⁻¹ range a band at 855 cm⁻¹ seen in the spectrum of DDVP was absent from that of the derivative, whilst a new band appeared at 745 cm⁻¹; and a strong absorption at 1,280 cm⁻¹ associated with P=O vibration was found in the spectrum of both DDVP and the derivative. The presence of a non-conjugated C=C bond was further supported by the strong absorption at 1,650 cm⁻¹ in infrared spectra both for DDVP and the another decomposed material, and by the chemical reaction with potassium permanganate in sodium carbonate where decoloration occurred by the derivative as well as by DDVP. These data suggested that both the
derivative and DDVP possessed C=C bond in the molecules. From these indications, it seems likely to assume that this compound is not DDVP, but some closed compound to DDVP.

**Discussion**

The pH value of the digestive juice of silkworm larva is about 9 in feeding period and much higher in fasting condition\(^1\), while that of the hemolymph of the larva is around 6.5. It is, therefore, reasonable to say that the conversion of trichlorofon is more rapid in the digestive juice than in the hemolymph owing to the difference of pH, as explained in the previous paper\(^2\).

In the present experiments, it was shown that trichlorofon converted into DDVP even near pH 7 in the digestive juice, while the formation of DDVP was not significant in the non-enzymic reaction at this pH. This result indicates a possibility that an enzymic action takes a part in the dehydrochlorination of trichlorofon.

The conversion of trichlorofon into DDVP in the digestive juice seemed to be carried mainly by a non-enzymic reaction in high alkaline conditions, but by an enzymic reaction in low alkaline or neutral conditions. Miyamoto\(^3\) pointed that trichlorofon was converted into DDVP by non-enzymic transformation in neutral and acidic conditions and the resulted DDVP worked as an inhibitor of acetylcholinesterase in the fly head in vitro. Under neutral or acidic condition, from our experimental results, it is, however, doubtful to participate significantly the non-enzymic reaction to the conversion of trichlorofon. Certainly, in the digestive juice of the silkworm larva, non-enzymic transformation may not be ignored, even if there should be the enzymic reaction. At any rate, in the digestive juice, trichlorofon applied orally can be transformed into DDVP under wide range of pH by both non-enzymic and enzymic actions. Studies on the enzyme which is responsible for the dehydrochlorination of trichlorofon in the digestive juice is now under way.

**Summary**

The non-enzymic or enzymic conversion of trichlorofon into DDVP in the digestive juice of the silkworm larva was studied by means of ultraviolet spectrophotometry, thin-layer chromatography, infrared spectrophotometry and some chemical reactions. Trichlorofon was rapidly converted into DDVP in a mild alkaline solution, non-enzymically, but very slowly in neutral or slightly acidic condition. On the other hand, in the digestive juice of the silkworm larva trichlorofon rapidly changed to DDVP under a broader pH range of conditions, even in neutral or slightly acidic. It was shown that around neutral conditions, the conversion in the digestive juice progressed mainly enzymically. The enzyme activity concerned this conversion was recognized in the biological fluid, the pH optimum being around 7 or slightly lower. Near pH 9, which is normal pH of the digestive juice of silkworm larva feeding mulberry leaves, both non-enzymic and enzymic reactions seemed to participate in the conversion of trichlorofon. Enzymic activity was negligible above pH 10. Using the column of DEAE-cellulose, the active protein fractions were obtained. The active protein could be separated from juice proteins on zymograms of paper electrophoresis. In addition to DDVP, another decomposed compound of trichlorofon was formed during the reaction and this compound was partly characterized.

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**Literature Cited**