On the Fate of Methanol and Ethanol in the Adult Larva of the Cotton Leaf Worm (Prodenia litura F.)

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The metabolic fate of C\textsuperscript{14}-methanol and 1-C\textsuperscript{14}-ethanol in the adult larva of Prodenia litura has been investigated in vivo. Following the intersegmental injection of C\textsuperscript{14}-methanol (1.2 mg/g. insect), about 70% of the radioactivity was recovered as C\textsuperscript{14}O\textsubscript{2} in the expired air, after 20 hours. Also, about one fourth of the administered dose was recovered as unchanged methanol, during the first six hours. Only a minute trace of C\textsuperscript{14}-formate was found in the excreta. It is suggested that a catalase-peroxide system is involved in the oxidation of methanol. Ethanol and its metabolites were eliminated at a slower rate than methanol. After 20 hours, C\textsuperscript{14}O\textsubscript{2} — being the major C\textsuperscript{14}-metabolite — accounted for 56% of the applied dose. In addition, there is at least one minor non-volatile C\textsuperscript{14}-metabolite eliminated in the excreta of ethanol-treated larvae.

A number of organophosphorus esters, which are widely used as insecticides possess O-methyl or O-ethyl ester linkages. In insects, such organophosphates may suffer O-alkyl ester cleavage, thus liberating the free alcohol. Whereas the degradation of methanol\textsuperscript{1} and ethanol\textsuperscript{2} has been extensively studied in mammals, yet the fate of these alcohols in insects is not quite clear. Recent studies on the metabolism of O,O-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate (Dipterex) in the adult Prodenia larva have shown that the enzymatically liberated methanol undergoes partly a process of oxidative degradation to give carbon dioxide\textsuperscript{3}. For completion of our knowledge on the fate of methanol, a closer investigation of its metabolism in Prodenia larva seemed desirable.

In the present work the "in vivo" degradation of C\textsuperscript{14}-methanol and ethanol-C\textsuperscript{14} has been investigated.

Materials and methods

In all experiments, 4% aqueous solution (w/v) of the radioactive alcohol \* was used (sp. activity of C\textsuperscript{14}-methanol: 3.7 x 10\textsuperscript{4} cpm/mg and that of ethanol-1-C\textsuperscript{14}: 4.4 x 10\textsuperscript{4} cpm/mg). Healthy adult larvae (5th—6th instars) of almost the same size and weight were carefully injected, intersegmentally, with the radioactive alcohol using a fine needle. Unless otherwise stated, the dose was 1.2 mg/g insect. Throughout this investigation, a pool of 20 g. insect has been unused for each experiment.

Respiratory C\textsuperscript{14}O\textsubscript{2} and unchanged alcohol: For the collection of the expired air, metabolic cages\textsuperscript{4} were used and the C\textsuperscript{14}O\textsubscript{2} was trapped by 1 N sodium hydroxide solution and determined as BaC\textsuperscript{14}O\textsubscript{3}. Respiratory C\textsuperscript{14}O\textsubscript{2} was similarly determined for insects injected with methanol solution containing acetanilide. For each gram insect, an aqueous solution of 1.2 mg C\textsuperscript{14}H\textsubscript{2}OH (44400 cpm) and 50 \mu g acetanilide was used.

To collect the liberated free alcohol (methanol or ethanol) metabolic cages similar to those used for the collection of the respiratory CO\textsubscript{2} were used. The expired air — produced in six hours — was allowed to pass through three bubblers containing 60 ml dry benzene; the first bubbler containing 0.5 ml of non-labelled alcohol.

The alcohol was isolated in the form of the red coloured methyl- or ethylester of 4'-nitroazobenzene-4-carboxylic acid, I\textsubscript{a} and I\textsubscript{b}, respectively.

Preparation of the azoester I

To 10 ml of the combined benzene solution, 0.1 g of 4’-nitroazobenzene-4-carboxyl chloride\textsuperscript{5} and 2 drops of dry pyridine were added. The reaction flask was stoppered and allowed to stand at room temperature for 48 hours. The reaction mixture was shaken with 50 ml 0.05 N sulphuric acid and the organic layer was washed till neutral with distilled water, dried over anhydrous sodium sulphate and concentrated to 2 ml. This solution was chromato-

\* Amersham, Buckinghamshire, England.

graphed on neutral aluminium oxide (activity III) and the azoester I was eluted with benzene-cyclohexane (1:1). I a and I b formed red crystals from benzene-cyclohexane; m.p. of I a 185–186° (Lit. m.p. 186–187°), and of I b 161–163° (Lit. m.p. 162–163°). The specific activity of the isolated pure azoester was calculated after its C\textsuperscript{14}-activity had been determined as described by Aronoff\textsuperscript{6}, using Van Slyke Folch reagent\textsuperscript{7}.

**Non volatile C\textsuperscript{14}-metabolites**

For the characterization of the non volatile C\textsuperscript{14}-metabolites, the excreta produced in 20 hours were extracted 8 times with chloroform (each time 50 ml) and the residue was further extracted with water (8 times; each with 50 ml). Samples from the aqueous and chloroform extracts were determined for their C\textsuperscript{14}-activity, as previously mentioned. These extracts were concentrated under vacuum and analysed for possible C\textsuperscript{14}-metabolites by paper chromatography. In case of methanol-treated larvae a part of the water extract was used for the detection of C\textsuperscript{14}-formate using the inverse isotope dilution technique\textsuperscript{8}. C\textsuperscript{14}-activity in the larva was also determined after 20 hours, according to the procedure of Aronoff\textsuperscript{6}.

**Radio-measurements**

All radioactivity measurements—determined as BaC\textsuperscript{14}O\textsubscript{3}—were counted in an end-window counter under uniform geometrical conditions and the measurements were corrected for background and self absorption. Paper chromatograms were radiometrically assayed using a manually operated device with a GM-tube connected to a scaler and the data were corrected only for background.

**Results**

C\textsuperscript{14}H\textsubscript{2}OH: Fig. 1 shows the cumulative elimination of C\textsuperscript{14}O\textsubscript{2} in the expired air during 20 hours. During the first 6 hours, the major part of C\textsuperscript{14}O\textsubscript{2} (about 66.5% of the applied dose) has been eliminated. This amount corresponds to 94.3% of the C\textsuperscript{14}O\textsubscript{2} recovered in 20 hours, and gives a mean value of 420 μmole/hour/100 g insect for the rate of transformation of methanol into carbon dioxide. During the last 14 hours the rate of elimination of C\textsuperscript{14}O\textsubscript{2} is remarkably decreased and the produced amount is only 5.7% of the total C\textsuperscript{14}O\textsubscript{2} eliminated during the period of experimentation (20 hours).

When acetanilide was injected with methanol, the amount of the expired C\textsuperscript{14}O\textsubscript{2} was significantly lowered (Fig. 2). During the first 5 hours the evolved C\textsuperscript{14}O\textsubscript{2} contributed about 40.5% of the applied dose, compared with 63% in non-treated larvae; thus effecting an inhibition percentage as high as about 36 per cent.

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\textsuperscript{6} S. Aronoff, "Techniques of Radiobiocchemistry", The Iowa State College Press (1957), Ames, Iowa.


\textsuperscript{8} A. Hassan and S. M. A. D. Zayed, Canad. J. Biochem. Physiol. 43, 1271 [1965].
When 24 mg C\textsuperscript{14}H\textsubscript{3}OH were injected into 20 g insect and the isolation of the azoester I\textsubscript{a} proceeded as described, 3.4 mg of pure crystalline ester were obtained which counted 300 cpm. On this basis, it could be calculated that 251 300 cpm (equivalent to 6.79 mg methanol) were eliminated as unchanged methanol. This amount contributed 28.2\% of the applied dose. In another experiment (28 mg methanol applied to 20 g insect), a total activity of 222 000 cpm was recovered. This corresponds to 21.4\% of the administered dose.

The total chloroform extracts contained 900 cpm (about 0.1\% of the applied dose). This amount corresponds to about 24 \mu g methanol. The aqueous extracts contained 3220 cpm (0.36\% of the applied dose). From this radioactivity only a small amount could be isolated as C\textsuperscript{14}-formate (about 0.02\% of the applied dose). Table 1 shows the \(R_f\)-values of C\textsuperscript{14}-metabolites obtained from chloroform and aqueous extracts. Apparently, both extracts contain the same substance.

<table>
<thead>
<tr>
<th>Extract</th>
<th>System</th>
<th>Metabolites of Methanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>A</td>
<td>0.26</td>
<td>0.7</td>
</tr>
<tr>
<td>Water*</td>
<td>A</td>
<td>0.26</td>
<td>0.7</td>
</tr>
<tr>
<td>Chloroform</td>
<td>B</td>
<td>0.63</td>
<td>0.82</td>
</tr>
<tr>
<td>Water*</td>
<td>B</td>
<td>0.63</td>
<td>0.80</td>
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The worms were found to contain negligible radioactivity after 20 hours (less than 2\%).

**Ethanol-1-C\textsuperscript{14}:** As in the case of methanol, the rate of elimination of respiratory C\textsuperscript{14}O\textsubscript{2} from ethanol-treated larvae, followed a 2-phased curve (Fig. 1). During 20 hours about 56.5\% of the administered radiodose was eliminated as C\textsuperscript{14}O\textsubscript{2}. After 4 hours, 43.5\% of the radioactivity could be recovered in the expired air, giving a value of 280 \mu mole/hour/100 g insect for the rate of transformation of ethanol to carbon dioxide.

The amount of unchanged ethanol produced from ethanol-treated larvae was relatively small. From 24 mg/20 g insect, only 61 500 cpm could be recovered in the azoester I\textsubscript{b}. This is equivalent to about 5.8\% of the administered dose. In a second experiment (28 mg/20 g insect) about 6\% of the applied dose could be recovered as unchanged ethanol.

After 20 hours from administration of ethanol, the worms were found to contain about 36.3\% of the original radioactivity. The radioactivity present in the aqueous and chloroform extracts accounted for 2.7\% and 0.3\% of the applied dose, respectively.

**Discussion**

The data presented in this investigation clearly show, that the adult larva of the cotton leaf worm eliminates methanol and methanol metabolites at an exceptionally high rate. After 20 hours, the larva was found to contain not more than 2\% of the applied dose. During the first six hours about 91\% of the applied C\textsuperscript{14}-activity could be recovered as C\textsuperscript{14}O\textsubscript{2} and unchanged methanol. The non-volatile C\textsuperscript{14}-metabolites, eliminated in the excreta contributed only about 0.5\% of the injected dose.

The origin of the recovered unchanged methanol or ethanol is uncertain. It may have been eliminated via the respiratory system and/or the excretory system. In this connection, a variable amount of methanol is known to be eliminated in different mammalian species (up to 20\%)\textsuperscript{1}.

The elimination of C\textsuperscript{14}O\textsubscript{2} from C\textsuperscript{14}-methanol-treated larvae is believed to proceed, as in mammals\textsuperscript{1}, through a multistep oxidation process:

\[
\text{CH}_3\text{OH} \rightarrow \text{HCHO} \rightarrow \text{HCOO}^- \rightarrow \text{CO}_2.
\]

The physiological oxidation of methanol in mammals has been a subject of much controversy. The early concept claimed that the peroxidative action of catalase is involved in this transformation\textsuperscript{9}. The alcohol dehydrogenase system was, however, reported to be the enzyme responsible for methanol oxidation in the monkey\textsuperscript{1}. Recent investigations\textsuperscript{10,11} provided support for the former view. TEPHY et al.\textsuperscript{10} gathered sufficient evidence to conclude that methanol is mainly metabolized by a catalase-peroxide

\textsuperscript{10} T. R. TEPHY, R. E. Parke, Jr., and G. J. MANNERING, J. Pharmacol. exp. Therapeut. 14, 3292 [1964].
system in the rat. Hassan et al.\textsuperscript{11} found that acetanilide inhibits methanol metabolism in mice, whereas the drug was without effect on ethanol oxidation and it was concluded that a peroxidative action, mediated by catalase, is involved in methanol oxidation. The role of acetanilide in inhibiting the alcohol oxidation is believed to be related to the depletion of the peroxide pool, available for catalase. Since administration of acetanilide to methanol-treated larvae resulted in an inhibition of the alcohol metabolism, it is suggested that the metabolic pathway of methanol in mice and \textit{Prodenia} larvae is practically the same. Should this view be accepted for the larva, the catalase-peroxide system would catalyse the oxidation of methanol to formaldehyde and formate to carbon dioxide. The oxidation of the aldehyde is probably mediated by an aldehyde oxidase\textsuperscript{3}. The isolation of a minute trace of C\textsuperscript{14}-formate supports the suggested chain for methanol metabolism. It is worth mentioning that evidence for the oxidative degradation of methanol in \textit{Prodenia} larva was first gained from the "in vivo" metabolism of \textit{O,O-C\textsuperscript{14}-dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate} (Dipterex)\textsuperscript{3}.

Compared with methanol, it appears that ethanol and its metabolites are eliminated at a slower rate. After 20 hours, the larvae still retain about 36\% of the injected dose; whereas the corresponding value for methanol-treated larvae did not exceed 2 per cent. Even after the elapse of 36 hours, the C\textsuperscript{14}-activity eliminated as C\textsuperscript{14}O\textsubscript{2} constituted 60\% and that retained by the larvae 25\% of the administered dose, respectively. The oxidation of ethanol to CO\textsubscript{2} is believed to proceed — as in mammals — through the intermediate formation of acetaldehyde and acetic acid (or active acetyl). Though it is almost certain that an ethanol dehydrogenase system catalyzes the conversion of ethanol to acetaldehyde in mammals\textsuperscript{2}, the identity of such enzyme system in \textit{Prodenia} larvae must await further experimentation. The oxidation of acetaldehyde to the acid is probably catalyzed by an aldehyde dehydrogenase\textsuperscript{3}. The nature of the minor non-volatile C\textsuperscript{14}-metabolites from methanol and ethanol is unknown.