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INHIBITION OF ENZYMIC OXIDATION OF INDOLE-3-ACETIC ACID BY METABOLITES OF THE INSECTICIDE CARBOFURAN

T. T. LEE and R. A. CHAPMAN

Research Institute, Agriculture Canada, University Sub Post Office, London, Ontario, Canada, N6A 5B7

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Key Word Index—Indole-3-acetic acid; IAA oxidase inhibitor; insecticide; carbofuran; benzofuran; peroxidase.

Abstract—Metabolites of carbofuran, a carbamate insecticide, inhibit the enzymic oxidation of indole-3-acetic acid. The metabolites differ in stability and effectiveness. 2,2-Dimethyl-7-hydroxy-2,3-dihydrobenzofuran represents one type which is broken down in the IAA oxidation reaction; thus the induced inhibition is limited by depletion of the the inhibitor. 2,2-Dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran represents the other type which is stable in the reaction; thus the inhibition is persistent. With both types of inhibitors the inhibition is reversible by higher substrate concentrations, but the Lineweaver–Burk plot is curvilinear suggesting the complex nature of competitive inhibition.

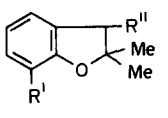
INTRODUCTION

In the study of the growth-regulating activity in plants of the insecticide carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl-7-*N*-methylcarbamate), it was found that three of its metabolites were more active than carbofuran itself and that the promotion of plant growth occurred only when indole-3-acetic acid (IAA) was present. The synergistic effect of these compounds with IAA on plant growth was correlated with their inhibitor activity in the enzymic degradation of IAA [1, 2]. Since carbofuran was rapidly degraded in plants [3] and the metabolites varied in growth-regulating activities, further studies were carried out to obtain a better understanding of the role of the metabolites in the enzymic oxidation of IAA. This report deals with the kinetic aspects of inhibition which differed with the metabolites.

RESULTS

Temporary inhibition

Among the carbofuran metabolites, 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (**3**) was more inhibitory to enzymic degradation of IAA than others [1, 2]. Even with **3**, however, the enzyme activity recovered after a



R^I = OCONHMe,	R^{II} = H	Carbofuran
R^I = OCONHMe,	R^{II} = OH	**1**
R^I = OCONHMe,	R^{II} = O	**2**
R^I = OH,	R^{II} = H	**3**
R^I = OH,	R^{II} = OH	**4**
R^I = OH,	R^{II} = O	**5**

lag period, the length of which was dependent on the inhibitor concentration (Fig. 1). For a 10-min period, **3** at 10 μM effectively inhibited IAA breakdown but the inhibition period was shortened with decreased concentrations. After the lag period, the rate of IAA degradation reached the same level as that of the control without the inhibitor. A similar pattern of inhibition was found with 2,2-dimethyl-3,7-dihydroxy-2,3-dihydrobenzofuran (**4**), carbofuran and 2,2-dimethyl-3-hydroxy-2,3-dihydrobenzofuranyl-7-*N*-methylcarbamate (**1**) although the effective concentration varied greatly. The latter was not inhibitory unless a high concentration, several-fold of that of IAA, was used. A lag period prior to enzymic oxidation of IAA induced by certain phenolics such as catechol and ferulic acid has been reported [4–7].

The results shown in Fig. 1 were obtained with the

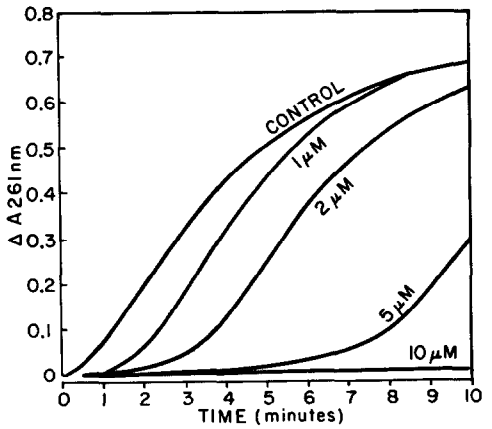


Fig. 1. Time course of inhibition of IAA degradation by 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (**3**). The composition of the reaction solution was 0.2 m M IAA, 0.1 m M each $MnCl_2$ and DCP, 25 m M K–Pi buffer, pH 5.9, 0.12 μg HRP and 1–10 μM **3**. The total vol was 3 ml.

inhibitor added before horseradish peroxidase (HRP). However, the lag period was greatly shortened when **3** was added a few sec after HRP (Fig. 2). Note that a 30-sec delay for the addition of the inhibitor reduced the lag from 18 min (not shown) to less than 30 sec. The lag was

also affected when 3 was mixed at the same time with HRP. It appears that a rapid IAA–enzyme interaction was responsible for the decreasing inhibitor activity of 3. The severe inhibition in treatment 7 (Fig. 2), in which IAA was mixed with a low concentration of HRP but

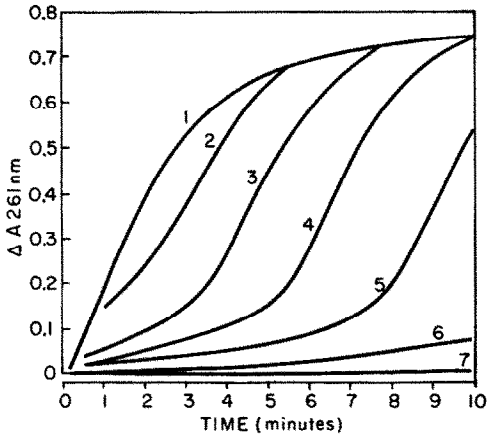


Fig. 2. Changes in lag period of IAA degradation by delayed addition of 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (3). 3 ml reaction solution contained the same components as that in Fig. 1 except that 3 was 20 μM. (1) control; (2) 3 added 30 sec after HRP; (3) 3 added 5 sec after HRP; (4) 3 added 3 sec after HRP; (5) 3 and HRP added and mixed at 0 time; (6) 3 added and mixed 2 min before HRP; (7) IAA mixed with HRP for 3 min, then 3 added and mixed, followed by $MnCl_2$ and DCP.

without 2,4-dichlorophenol and $MnCl_2$ for 3 min before addition of 3 further suggests that an active IAA degradation system was related to the loss of inhibitor activity of 3. This could mean that 3 either was no longer inhibitory to the transformed HRP known to exist during

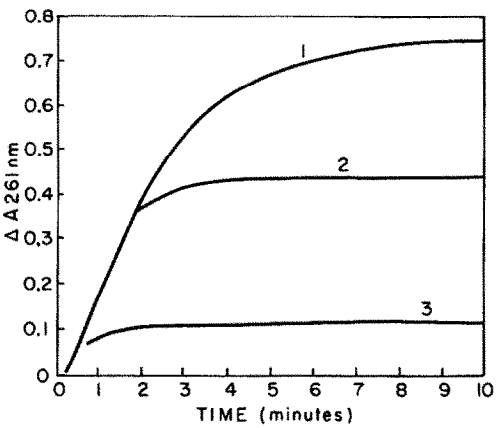


Fig. 3. Inhibition of IAA degradation by high concentrations of 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (3) added at a later stage of reaction. 3 ml reaction solution contained the same components as that in Fig. 1 except that 3 was 0.2 mM. (1) control; (2) 3 added 90 sec after HRP; (3) 3 added 30 sec after HRP.

IAA degradation [8–10] or was broken down in the reaction. From the inhibition curves (Fig. 2) apparently 3 was also inhibitory in the early stage of the reaction though with shortened lag period. To know whether it was an effective inhibitor in the later stage of IAA oxidation, a high concentration of 3 which was 10 times that used for Fig. 2 was added 30 sec after HRP or later. A part of the result is shown in Fig. 3. Evidently 3 was also inhibitory to the reaction in a later stage. Thus it appears that the inhibitor was effective on different forms of HRP produced in the course of IAA degradation.

Breakdown of Inhibitors

Whether metabolite 3 was truly broken down in the reaction as suggested in Figs 1 and 2 was tested spectrophotometrically and chromatographically. This compound has a distinct absorption peak at 202 nm but the reaction solution for IAA oxidation interferes at this wavelength. This difficulty was overcome by measuring the difference absorbance using an active IAA degradation system as reference. A reaction solution and an inhibitor concentration were so chosen that when the inhibitor was added 1 min after HRP the inhibition was minimal and the rate of IAA breakdown was similar to that in the reference cuvette. The results are shown in Fig. 4. The decrease in A_{202nm} represents largely the

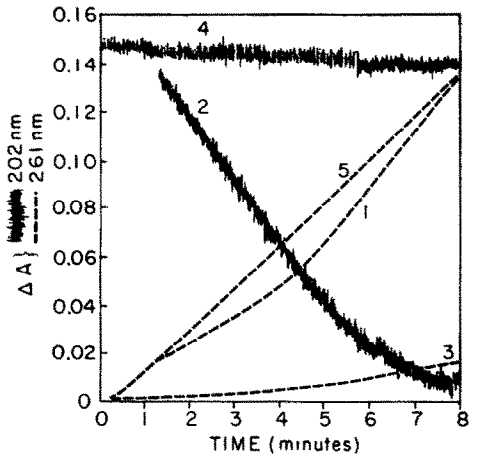


Fig. 4. Breakdown of 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (3) during IAA oxidation reaction. The composition of the reaction solution was 67 μM IAA, 6.7 μM each of $MnCl_2$ and DCP, 30 mM K–Pi buffers, pH 5.9, 0.25 μg HRP with or without 3.3 μM 3. The total vol was 3 ml. (1) 3 added 1 min after HRP showing the rate of IAA oxidation; (2) same treatment as 1 showing the rate of 3 breakdown; (3) and (4) 3 added before HRP; (5) control without 3.

disappearance of 3. When IAA degradation was largely inhibited by 3 added before HRP (Fig. 4-3), A_{202nm} decreased only slowly (Fig. 4-4). However, A_{202nm} decreased rapidly when the inhibitor was added 1 min after HRP (Fig. 4-2). Under this condition the rate of IAA degradation was rapid with minimal interruption after the inhibitor was added (Fig. 4-1). Evidently 3 was rapidly broken down in the active IAA degradation enzyme system. On the other hand, preincubation of the inhibitor with IAA, $MnCl_2$ or HRP alone did not lower its inhibitor activity. A similar result was found with the 3,7-dihydroxy-derivative (4).

The breakdown of these inhibitors was confirmed by TLC and GLC analyses. There was very little breakdown in the early stage of the lag period and *ca* 50% breakdown at the end of the lag period, followed by a rapid rate

of breakdown of both inhibitor and IAA. The inhibitors were stable in an inactive IAA oxidase system in which HRP or IAA was missing.

Thus it is clear that the recovery of IAA oxidase activity after a lag period induced by the carbofuran metabolites was due to breakdown of the metabolites in the reaction.

Persistent inhibition

In contrast to the inhibition curves which showed recovery of IAA oxidase activity after a lag period (Fig. 1), the inhibition by two other carbofuran metabolites, 2,2-dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran (**5**) and 2,2-dimethyl-3-keto-2,3-dihydrobenzofuranyl-7-*N*-methylcarbamate (**2**) was different; one example is shown in Fig. 5. The rate of IAA degradation was inhibited to various degrees by different concentrations of **5** and the

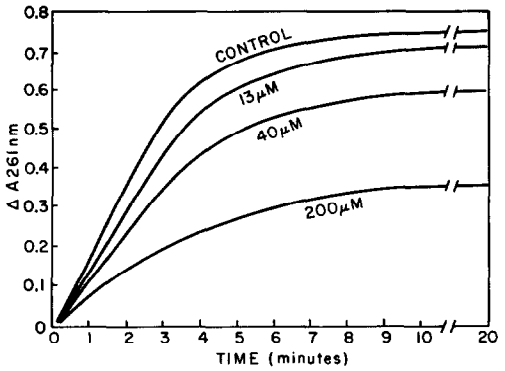


Fig. 5. Time course of inhibition of IAA oxidation by 2,2-dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran (**5**). Composition of the reaction solution was 0.2 mM IAA, 0.1 mM each of $MnCl_2$ and DCP, 25 mM K–Pi buffer, pH 5.9, 0.25 μg HRP and 13–200 μM **5**. The total vol was 3 ml.

inhibition was consistent throughout even with a low inhibitor concentration. A similar result was found with metabolite **2** although higher concentrations were required. Comparison of Figs 1 and 5 also shows the difference in effective concentration between metabolites **3** and **5**. Experiments similar to those shown in Fig. 4 demonstrated that the keto-derivatives were not broken down in the reaction in which IAA was oxidized. The stability of the compounds contributed to the persistence in inhibition of IAA degradation.

Experiments were also done with delayed addition of the keto-derivatives to the active IAA degradation system. Figure 6 shows that the degree of inhibition was the same for all treatments with **5** added at different times after HRP. Rapid recovery of enzyme activity by delayed addition of inhibitor as seen in Fig. 2 was not found with this metabolite. The results provided additional proof for the stability of the inhibitor in active IAA oxidase system and for its effectiveness as inhibitor for IAA degradation in different stages of reaction.

Effect of substrate concentration

The lag period for IAA degradation in the presence of metabolite **3** could be shortened by increasing the IAA concentration (Fig. 7). However, a complete reversal was not observed. One difficulty was that high concentrations of IAA showed substrate inhibition. When a broad range of IAA concentration was used, the Lineweaver–Burk plot was not linear (Fig. 8). In addition to inhibition by high IAA concentrations, the reaction rate also was lower

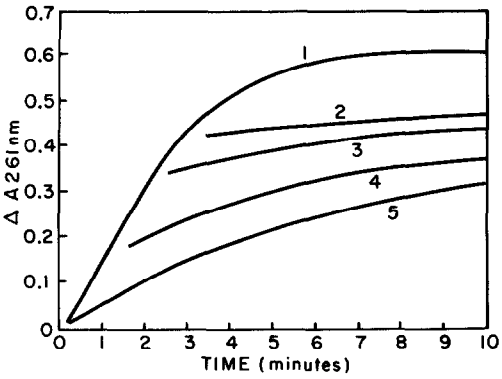


Fig. 6. Effect of delayed addition of 2,2-dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran (**5**) on inhibition of IAA degradation. 3 ml reaction solution contained the same components as that in Fig. 1 except that **5** with a concentration of 0.13 mM was used as the inhibitor. (1) control; (2), (3), (4) **5** added 3, 2, and 1 min after HRP respectively; (5) **5** added before HRP.

than usual at the low end of the concentration range. With metabolite **3** acting as inhibitor the plot was further curved especially with low IAA concentrations. Apparently the inhibition was not fully competitive. There was

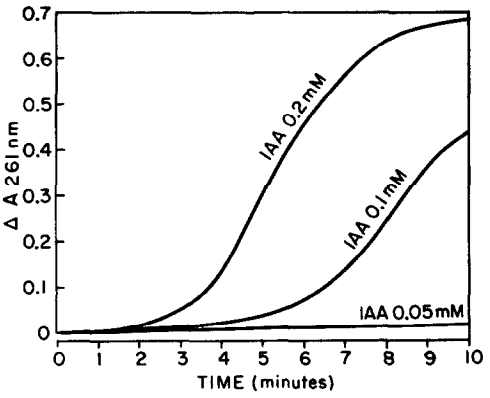


Fig. 7. Reversal of 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran induced inhibition by higher concentrations of IAA. 3 ml reaction solution contained the same components as that in Fig. 1 except that 10 μM of **3** and varied concentrations of IAA were used.

a similar IAA concentration effect upon the inhibition of IAA degradation induced by the keto-derivatives.

DISCUSSION

From the results reported it is evident that among the carbofuran metabolites there were two types of inhibitors for IAA degradation catalyzed by peroxidase. They differed in stability and reactivity. 2,2-Dimethyl-7-hydroxy-2,3-dihydrobenzofuran (**3**) represented one type which was active at low concentrations but easily broken

down in the active IAA degradation system. The keto-derivatives such as 2,2-dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran (**5**) represented the other type which was active at relatively high concentrations and was stable. The latter is particularly interesting because it is different from other known phenolic inhibitors such as ferulic acid and scopoletin which were degraded in IAA oxidase reaction [7, 11].

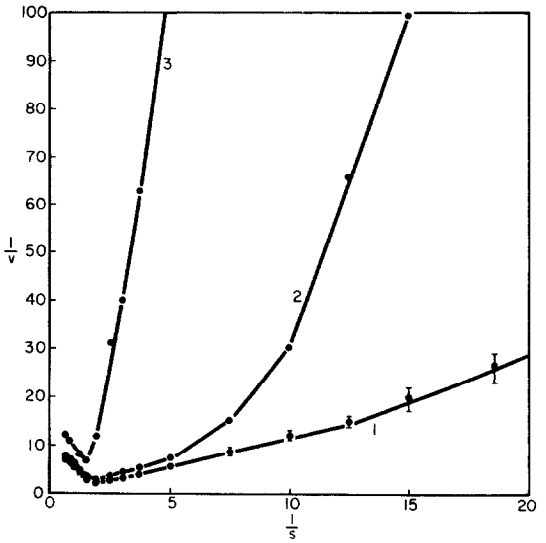


Fig. 8. Lineweaver–Burk plot of IAA concentration against reaction rate with and without 2,2-dimethyl-7-hydroxy-2,3-dihydrobenzofuran (**3**). Composition of the reaction solution was 0.033–1.4 mM IAA, 0.2 mM DCP, 0.2 mM $MnCl_2$, 20 mM K–Pi buffer, pH 5.9, 2.5 μg HRP with or without **3**. The reaction rate as an increase in A_{261nm} in the first min was used. (1) control without **3**; (2) with 1.7 μM of **3**; (3) with 6.7 μM of **3**. V = ΔA_{261}. min^{-1}, S = IAA conc in mM.

Because of the partially competitive nature, the inhibition of IAA degradation induced by the carbofuran metabolites was incomplete. Therefore, IAA degradation occurred at a slow rate during the inhibitor-induced lag period. This may not be detectable by methods of low sensitivity. It is for this reason that the metabolites **3** and **4** were continuously broken down even in the lag period. As the inhibitor concentration decreased, the rate of IAA degradation increased, which in turn accelerated further destruction of the inhibitor until its concentration was sufficiently reduced, then the rate of IAA oxidation rose rapidly to the level found without inhibitor. Breakdown of scopoletin, a phenolic inhibitor of IAA oxidation, in a IAA-HRP system has been reported by Sirois and Miller [11]. A similar result was reported for ferulic acid by Gelinas [7].

It has been known that in the process of IAA degradation HRP underwent a series of changes [8–10]. The progressive reduction in the length of lag period by delayed addition of metabolite **3** (Fig. 2) suggests that the rate of enzyme transformation might be a key factor for the lag. Miller *et al.* [12] have reported that oxidation of scopoletin in IAA-HRP system required a longer time than in H_2O_2–HRP system because the initial reduction of HRP to HRP-III was the rate-limiting step for the IAA-promoted reaction [11, 13]. Gelinas has proposed HRP-1 and HRP-II as effective enzyme forms for the breakdown of ferulic acid [7]. From the data reported above it is difficult to ascertain which enzyme forms were dominant at a specified time, but it is plausible that transformed enzymes were required for the breakdown of metabolites **3** and **4**. Metabolite **3** did not react with the IAA degradation products nor was it broken down by HRP in the absence of IAA. For the breakdown of **3** and **4** IAA played a dual role; it first initiated the transformation of HRP to HRP-III and second served as substrate for HRP-II and HRP-I.

The inhibition of IAA oxidation by carbofuran metabolites was reversible by higher concentrations of IAA (Fig. 7) but the Lineweaver–Burk plot shows that the nature of inhibition was complex (Fig. 8). A similar result has been reported for scopoletin (11). The kinetic behaviour of inhibition induced by the carbofuran metabolites may reflect a partially competitive type of inhibition where the inhibitor and IAA had separate sites. Alternatively, it may reflect a complex nature of different enzyme forms which participated in IAA oxidation but differed in affinities for inhibitor and IAA. The latter may also explain the nonlinearity at the right side of the Lineweaver–Burk plot for the control without inhibitor. The nature of inhibition by the carbofuran metabolites remains to be investigated.

It has already been shown that the inhibition of IAA oxidation by carbofuran metabolites was correlated with promotion of plant growth [1, 2]. The difference in stability and relative activity may have further implications. According to Metcalf *et al.* [3] the metabolites **3** and **4** were found only in trace amounts in cotton and corn tissues after feeding labelled carbofuran. The preference of these two metabolites over others for the IAA oxidase system and their instability in the reaction may contribute to the low residual levels in plant tissues.

EXPERIMENTAL

Horseradish peroxidase (EC 1.11.1.7, Sigma type VI) with a RZ of 2.9 was used. When low enzyme concentrations were used 2,4-dichlorophenol (DCP) and $MnCl_2$ were added to accelerate the reaction. The composition of reaction solns are given in the Figs. The reaction was carried out at 37° and the rate was recorded automatically with a spectrophotometer. The rate of IAA degradation was estimated by the increase in A_{261nm}, a measure of formation of IAA degradation products [14]. Similar results were obtained at 25°. Breakdown of carbofuran metabolites was detected by spectrophotometry, TLC and GLC. For spectrophotometric detection difference spectra were measured with a reference so chosen that the interference from IAA oxidation products was minimal. The details are given in the text. For TLC and GLC analyses the metabolites were extracted 3 × from the reaction soln with purified Et_2O, which was combined and concentrated. Si gel plate was used with Et_2O–hexane (3:1) as the solvent system [3]. After development the metabolites were detected by spraying first with 15% KOH followed by 0.1% Gibb's reagent [15]. For GLC the metabolites were reextracted with C_6H_6, then treated with heptafluorobutyric anhydride at 95–100° for 1 hr [16]. Analyses were done with a 5% OV-1 on 100/120 Varaport 30 column pretreated with Carbowax 20M (17) and a gas chromatograph equipped with ^{63}Ni EC. 2,2-Dimethyl-3-keto-7-hydroxy-2,3-dihydrobenzofuran (**5**) was prepared from 3-keto-carbofuran (**2**) as described in ref. [3]. 2,2-Dimethyl-3,7-dihydroxy-2,3-dihydrobenzofuran (**4**) was prepared by hydrolysis of 3-hydroxy-carbofuran in 5% aq KOH at room temp for 24 hr. The aq hydrolysate, after extracted with Et_2O to remove any neutral material, was acidified and extracted with Et_2O. Purity of the compounds was

verified by GLC. Carbofuran and three other derivatives were gifts of Niagara Chemical Division, F.M.C. Corporation, Middleport, NY. Horseradish peroxidase was purchased from Sigma.

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