The Herbicide *Command* Does Not Inhibit the Prenyl Diphosphate-Forming Enzymes in Plastids

Manfred Lützow, Peter Beyer, and Hans Kleinig
Institut für Biologie II, Universität Freiburg, Schänzlestraße 1, D-7800 Freiburg, Bundesrepublik Deutschland

Z. Naturforsch. **45c,** 856–858 (1990); received April 6, 1990

Command, Bleaching Herbicide, Prenyl Diphosphates, Carotinoid Formation, Plastids

The herbicide *Command* (2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone) does not affect the *in vitro* activities of the plastid enzymes catalyzing the steps leading from isopentenyl diphosphate to geranylgeranyl diphosphate and phytoene, *i.e.* the isopentenyl diphosphate isomerase, prenyl transferase and phytoene synthase. The extractable activities of these enzymes in herbicide-treated seedlings are also not affected. Nevertheless, the synthesis of chlorophylls and carotenoids in treated seedlings is severely inhibited *in vivo*. The mode of action of *Command* remains still unknown.

Introduction

Command (FMC 57020, 2-(2-chlorophenyl)-methyl-4,4-dimethyl-3-isoxazolidinone) is a commercial pre-emergence herbicide for weed control in soybeans [1]. Based on the results of *in vivo* experiments, it was assumed "that one or more components" of the photosynthetic electron transport chain are the targets of this substance [2]. The same authors also observed a changed Shibata shift and mentioned the possibility that the formation of geranylgeranyl diphosphate and thus the phytylation of chlorophyllide might be inhibited [2]. These assumptions were confirmed by others using various objects (plants, algae): inhibition of the isoprenoid pathway *in vivo* and *in vitro* between the formation of isopentenyl diphosphate and the consumption of farnesyl or geranylgeranyl diphosphate was reported [3, 4]. The I_{50} values measured suggested *Command* to be an effective *in vitro* inhibitor of the formation of terpenoid compounds [4].

Since the chromoplast system from daffodil flowers is well established for the investigation of the pathway leading to carotenes [5–7] and has been successfully used for testing herbicides affecting the desaturation of phytoene [8], we were prompted to search in this system for the target of *Command*. Our main interest was the question whether this herbicide affects the isopentenyl diphosphate isomerase (EC 5.3.3.2) or the following enzyme of the pathway, the prenyl transferase (EC 2.5.1.29). Additional experiments using mustard seedlings and etioplasts prepared from such seedlings grown under far red light, which also exhibit high carotenogenic activities *in vitro* [9], were included in these investigations.

Methods

The effect of *Command* on the greening of seedlings was tested with mustard (*Sinapis alba* L.) grown under day light and watered with 4.2 and 42 μM (1 and 10 ppm) of the herbicide. The herbicide was dissolved in methanol and added to the supplied water without intermediate dilution. Controls contained only methanol. Growth conditions were chosen as described [10]. After four days of growth the cotyledons of the seedlings were harvested and their fresh and dry weight (after lyophilization) determined. Chlorophyll and carotenoid contents were also measured [11]. Etiolated mustard seedlings were obtained by growth under far red light [12], and etioplasts from such seedlings were prepared as described earlier [9].

Chromoplasts and chromoplast stroma from daffodil flowers were prepared as described previously [6]. A preparation of the isopentenyl diphosphate isomerase free from prenyl transferase and phosphatase activities was obtained by gel chromatography [6]. Isomerase assays [6] were performed in the presence of *Command* and other inhibitors (dimethylaminoethyl diphosphate, a

Reprint requests to H. Kleinig.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0700–0856 $ 01.30/0

transition state analogue in the isomerization of isopentenyl diphosphate to dimethylallyl diphosphate, and fluoromethylbutenyl diphosphate [13], both kindly supplied by C. D. Poulter, Salt Lake City, U.S.A.). All assays were preincubated for 30 min in the presence of inhibitors prior adding the substrate. The prenyl transferase (geranylgeranyl diphosphate-forming) and the subsequent phytoene synthase of the pathway were assayed together in the stroma as reported [6].

Results and Discussion

When seedlings of *Sinapis alba* were grown in the presence of *Command*, the formation of chlorophyll and carotenoids in their cotyledons was severely impaired, even at remarkably low levels of the herbicide (Table I). Fresh and dry weight of the cotyledons, on the other hand, were not affected. These results are comparable to those obtained earlier for primary leaves of seedlings from *Vigna unguiculata* [2].

In vitro, however, using isolated plastids – etioplasts from mustard seedlings and chromoplasts from daffodil flowers – no inhibitory effects of *Command* on carotenogenic enzymes could be detected. In Tables II and III results obtained with chromoplasts are shown. *Command* did not show any inhibition of the isopentenyl diphosphate isomerase, not even at concentrations of 20 mM (Table II). Established inhibitors of this enzyme such as dimethylaminoethyl diphosphate and fluoromethylbutenyl diphosphate [13], on the other hand, were very effective (Table II). In Table III results with the enzymes prenyl transferase and phytoene synthase in a combined test are shown. These enzymes catalyze the reactions following the

Table II. Influence of *Command* and two other inhibitors on the isopentenyl diphosphate isomerase isolated from daffodil chromoplasts. The activity of the enzyme was determined by acidic hydrolysis of the dimethylallyl diphosphate formed from the ^{14}C-labeled substrate isopentenyl diphosphate and its separation from the unreacted substrate in a two-phase partition system [6].

Incubation conditions	Inhibitor concentration	Enzyme activity [%]
Control	–	100
Control + 10 µl methanol	–	93
Command	0.2 mM	104
Command	2 mM	97
Command	20 mM	94
Dimethylaminoethyl diphosphate	1 µM	0
Fluoromethylbutenyl diphosphate	1 µM	10

Table III. Influence of *Command* on the synthesis of phytoene in the stroma from daffodil chromoplasts measured in a combined test for prenyl transferase (geranylgeranyl disphosphate-forming) and phytoene synthase. In these experiments ^{14}C-labeled isopentenyl diphosphate plus non-labeled dimethylallyl diphosphate were supplied as substrates in order to circumvent the isomerase. Without dimethylallyl diphosphate the same results were obtained, except in incubations in the presence of fluoromethylbutenyl diphosphate, which is a strong inhibitor of the isomerase (see Table II).

Incubation conditions	Inhibitor concentration	Phytoene synthesis [% of control]
Control	–	100
Control + 10 µl methanol	–	105
Command	2 mM	107
Fluoromethylethyl diphosphate	0.1 mM	23

Table I. Inhibition of pigment synthesis in mustard seedlings by the herbicide *Command*. After four days of growth in the presence of the herbicide in a concentration of 4.2 µM (1 ppm) and 42 µM (10 ppm), fresh weight (FW) and dry weight (DW) of the cotyledons (cot) as well as their total chlorophyll and total carotenoid contents (see Methods) were determined.

Command [µM]	Fresh weight [g/20 cot]	Dry weight [g/20 cot]	Chlorophyll [nmol/g FW]	Carotenoids [nmol/g DW]
0	0.49	0.070	185	53
4.2	0.50	0.068	42	14.6
42	0.49	0.080	15	6

isomerase reaction in the carotenogenic pathway. Phytoene synthesis was not affected by the herbicide. The fluorinated analogue of isopentenyl diphosphate, however, was also an inhibitor of one or both of these enzymes, although at higher concentrations than with the isomerase. (This was not further analyzed.)

The conflict between the results obtained *in vivo* (Table I) and *in vitro* (Tables II/III) led us to the assumption that the extractable activities of the enzymes of the pathway leading from isopentenyl diphosphate to phytoene could be affected by the herbicide. In order to test this hypothesis, mustard seedlings were grown under far red light in the presence of 42 μM (10 ppm) *Command*. After four days of growth, etioplasts were isolated from the cotyledons of these treated seedlings and the specific activities of enzymes were determined and compared with those of control seedlings, which were grown under the same conditions, but in the absence of the herbicide. No differences could be observed between the specific activities of the enzymes in the plastids from the two types of seedlings (Table IV). This shows that the extractable activities of carotenogenic enzymes are apparently not affected in plants treated with *Command*.

The results presented in Tables I to IV show that *in vitro* the herbicide *Command* does not affect the synthesis of phytoene from isopentenyl diphosphate, *i.e.* the isopentenyl diphosphate isomerase, prenyl transferase and phytoene synthase. This is in contrast to earlier investigations [3, 4], where I_{50} values in the range of 10 to 50 μM were reported for the *in vitro* formation of phytoene, phytol and squalene in a cell-free spinach system and similar values for the *in vitro* formation of phytoene and squalene in *Phycomyces* and of kaurene in *Fusarium*. We can not explain this discrepancy at the moment, which is quite remarkable, since the spinach system is comparable to our daffodil and mustard systems. Even in the millimolar range we could not observe any inhibition. *In vivo*, on the other hand, *Command* strongly impairs chlorophyll and carotenoid accumulation in mustard seedlings. This is in accordance with earlier findings in *Vigna unguiculata* [2]. The extractable activities of the enzymes isopentenyl diphosphate isomerase, prenyl transferase (geranylgeranyl diphosphate-forming) and phytoene synthase, however, are not affected in treated seedlings. This also means that geranylgeranyl diphosphate, the precursor of the phytol side chain of the chlorophylls, must be available. Thus, the mode of action and the target of this herbicide may be in a regulatory step under cellular conditions, but this question still remains open.

Table IV. Specific activities of isopentenyl diphosphate isomerase and phytoene synthesis (prenyl transferase plus phytoene synthase) in the stroma of etioplasts isolated from cotyledons of mustard seedlings grown in the absence and presence of the herbicide *Command* (42 μM = 10 ppm). Enzyme activities were measured as described [6].

	Protein [mg · ml^{-1}]	Isopentenyl diphosphate isomerase [pkat · mg^{-1}]	Phytoene synthesis [pkat · mg^{-1}]
Control	0.95	1.06	0.070
Command (42 μM)	0.84	1.27	0.063

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

[1] T. R. Warfield, D. B. Carlson, S. K. Bellman, and H. L. Guscar, Weed Sci. Soc. Amer. (WSSA), Abstr. **25,** 105 (1985).
[2] S. O. Duke and W. H. Kenyon, Pestic. Biochem. Physiol. **25,** 11–18 (1986).
[3] G. Sandmann and P. Böger, Z. Naturforsch. **41c,** 729–732 (1986).
[4] G. Sandmann and P. Böger, Z. Naturforsch. **42c,** 803–807 (1987).
[5] P. Beyer, K. Kreuz, and H. Kleinig, Planta **150,** 435–438 (1980).
[6] M. Lützow and P. Beyer, Biochim. Biophys. Acta **959,** 118–126 (1988).
[7] P. Beyer, M. Mayer, and H. Kleinig, Eur. J. Biochem. **184,** 141–150 (1989).
[8] M. P. Mayer, D. L. Bartlett, P. Beyer, and H. Kleinig, Pestic. Biochem. Physiol. **34,** 111–117 (1989).
[9] F. Lütke-Brinkhaus and H. Kleinig, Planta **170,** 121–129 (1987).
[10] T. Reiß, R. Bergfeld, G. Link, W. Thien, and H. Mohr, Planta **159,** 518–528 (1983).
[11] H. Lichtenthaler, Meth. Enzymol. **148,** 350–382.
[12] H. Mohr, Z. Pflanzenphysiol. **54,** 63–68 (1966).
[13] M. Muehlbacher and C. D. Poulter, Biochemistry **27,** 7315–7318 (1988).