The hybrid male sex-comb patterns are intermediate between those of either parental species, with some maternal bias except for hybrids of *malerkotliana* with *bipectinata* and with *parabipectinata*, which are either intermediate or like *malerkotliana*. This indicates some dominance of *malerkotliana* genes.

The best two discriminators in the analyses involving hybrids and their parents were the same as those which best discriminated the parental species from one another, except in two cases as indicated in table 2.

It is concluded that male sex-comb pattern, as defined by the numbers of teeth in each row of the sex-comb, is useful for distinguishing males within the *D. bipectinata* complex. It is preferred to the alternative of total sex-comb teeth number[2] because it classifies all possible hybrids with better than chance accuracy. Sex-comb patterns are relevant to a behavior genetic analysis of species-specific behaviors within this complex, which is in progress in this laboratory. Since the species occur sympatrically over parts of their ranges[4] and hybridization occurs in the wild[6], the use of sex-comb patterns for identifying species and hybrids is of general interest.

* Acknowledgments. We thank L. Anderson and P. Rowbury for technical and research assistance, Dr I. Bock for providing fly stocks and for his continued interest and helpful discussions, and the Australian Research Grants Scheme for financial assistance.

1 Bock, I. R., Syst. Entomology *5* (1980) 341.
2 Crossley, S. A., and Taylor, I., Experientia *41* (1985) 103.
3 Gupta, J. P., Experientia *29* (1973) 224.
4 Bock, I. R., Aust. J. biol. Sci. *31* (1978) 197.
5 Colgan, P. W., (ed.), Quantitative Ethology, p. 248. John Wiley publ., New York 1978.
6 Gupta, J. P., Dwivedi, Y. N., and Singh, B. K., Experientia *36* (1980) 290.

0014-4754/85/081072-03$1.50 + 0.20/0

Ecdysis inhibition in *Acrolepiopsis assectella* larvae by digitonin: antagonistic effects of cholesterol

C. Arnault and B. Mauchamp*

Lab. Biométrie, Université Claude Bernard, Lyon I, F-69622 Villeurbanne Cedex (France), and Lab. Phytopharmacie, INRA, F-78000 Versailles (France), 2 August 1984

Summary. Digitonin has a toxic effect on *Acrolepiopsis assectella*, inhibiting development and molting of young larvae fed on a semi-synthetic diet. This effect depends on concentration. It does not occur when cholesterol is added to the diet.
Key words. Ecdysis; saponins; digitonin; cholesterol; lepidoptera; *Acrolepiopsis assectella.*

Studies of *Acrolepiopsis assectella* showed that larvae fed on leek flowers (*Allium porrum*) or on a diet containing dry leek flowers, were unable to molt after normal apolysis. They could not remove their head capsules and their trunk exuviae[1]. Preliminary experiments to investigate the biologically active principle led us to isolate a steroidal saponin close to digitonin. Structural studies of this saponin are being carried out, with Dr Harmatha in Prag[2]. Since saponins are known to produce a significant reduction in plasma cholesterol concentration in various animals[3,4], interference with insect development was tested. We report the toxic effect of digitonin on *A. assectella* and the antagonistic effects of cholesterol. Ecdysis inhibition will be compared with similar effects obtained with different compounds.

Materials and methods. The methods of breeding *A. assectella* Zell. and the preparation of artificial diets have been previously described[1]. In our experiments we used 25 mm OD, 60 mm long glass test tubes. Each tube contained 5 ml of the artificial medium, and was closed with carded cotton. After solidification and cooling, the nutrient medium in each tube was inoculated with 50 3-day-old eggs; the larvae hatched with 24 h; breeding temperature was 25 °C, photophase 14 h, relative humidity 60–65%.

Digitonin used in the test was a commercial compound purchased either from Merck (Rahway, New Jersey, USA), or from Nativelle (Paris, France). In fact this commercial compound contains only 80% of digitonin. In preliminary experiments digitonin was purified on TLC (silica plates, butanol/ethyl acetate/water: 4:1:5) and the biological activity of other saponins; tigonin, gitonin and minor saponis, was tested. These experiments have shown that only digitonin was biologically active. Further experiments were performed with commercial products but the values reported correspond to those of pure digitonin.

The graded amounts of digitonin were added to the diet in water

Table 1. Larvae of *A. assectella* killed at the first instar or during molting, in diets containing various concentrations of digitonin. L1 = number of newly hatched larvae

Digitonin (ppm)	Total L1 hatched	Dead larvae 1st instar	Molting number 1	2	3	4
0	336	3				
200	304	26		1		
400	306	47		11	1	
600	686*	329	7	80	6	2
800	193**	142	1	26	2	
1000	312	242	4	49	5	2
Total	2137	789	12	167	14	4
% of dead larvae during molting			6.1	84.8	7.1	2

*16 replicates, **6 replicates.

Table 2. Decreasing sensitivity of *A. assectella* to digitonin with increasing age of larvae: 30 larvae of each instar transferred from standard diet to standard diet (control), or to 1000 ppm digitonin-containing diet. Number of dead larvae

Number of instar transferred	Control	Digitonin-fed without symptom	Molting number 2	3	4	Total
1st	7	18	10	1	0	29 (97%)
2nd	2	12	1	9	5	27 (90%)
3rd	2	11		1	9	21 (70%)
4th	3	10			3	13 (43%)
5th	0	5				5 (17%)

solution, which was pipetted into the test tubes in constant 0.5 ml amounts. After addition of 4.5 ml of the warm nutrient medium, the content of each tube was thoroughly mixed. In experiments with cholesterol, powder was added separately, just before solidification of the medium.

Each concentration of digitonin and digitonin-cholesterol was repeated eight times. Newly hatched larvae which had penetrated into the diet (L1) were considered for population evaluation. Larval mortality was estimated according to the formula: $\frac{L_1-N}{L_1} \times 100$ in which N is the number of pupae. Homogeneity of each result was calculated by the χ^2 test.

Results. Digitonin effects on larval development. The relation between digitonin concentration in the diet and larval mortality is shown in figure 1. The cumulative distribution of toxicity of digitonin in *A. assectella* is plotted as a sigmoid curve. The most reliable value is that corresponding to 50% kill, i.e. lethal dose LD 50, estimated at 570 µg of pure digitonin in 1 ml of the diet (570 ppm).

Two categories of pathophysiological syndromes were observed: 1) mortality of the newly hatched first instar larvae and 2) exdysial failure in the 2nd or 3rd larval instars. The first category can be related to an antifeeding effect, and closely resembled acute toxicity. The second category is shown in figure 2. Larvae remained enclosed within the old exuviae. Either they were unable to shed the old cuticle from the thorax and the abdomen, or they showed old head capsule permanently attached to the newly formed head. The cumulative distribution of ecdysial failures and appearance of the second category effects are shown in figure 3. We noticed that, even at high concentrations, only 20% of killed larvae revealed this syndrome. Digitonin concentration producing 50% ecdysial failure was also 570 ppm. Mortality distribution according to developmental stage and digitonin concentration in the diet is reported in table 1. The critical developmental stage at which those syndromes most frequently occurred was ecdysis from second to third larval instar. No

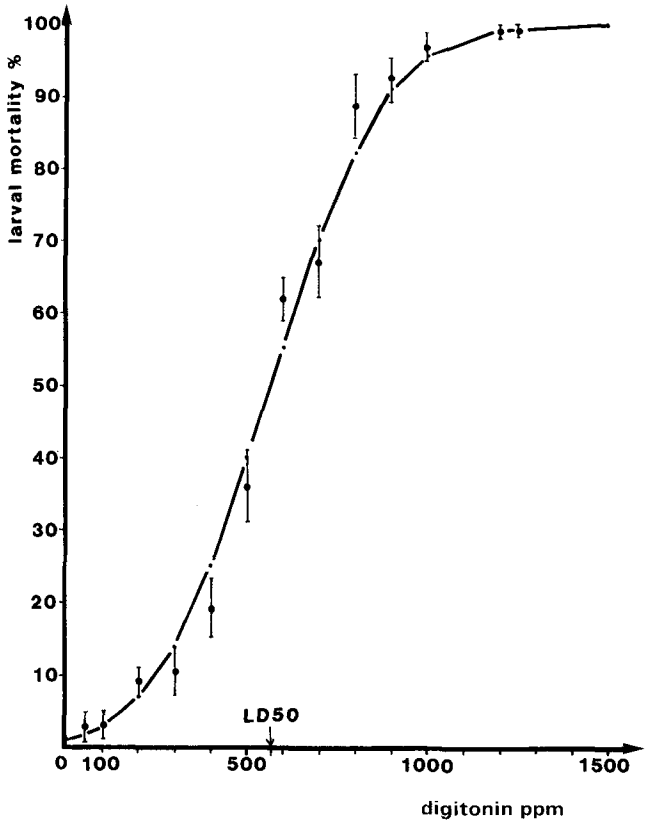

Figure 1. Dose-reponse relationship expressed by mortality of *Acrolepiopsis assectella* larvae reared on artificial diet containing digitonin. Each point is a mean of 8 replicates; vertical strikes are for SEM; sigmoid curve is adjustment to normal cumulative distribution; arrow indicates LD 50.

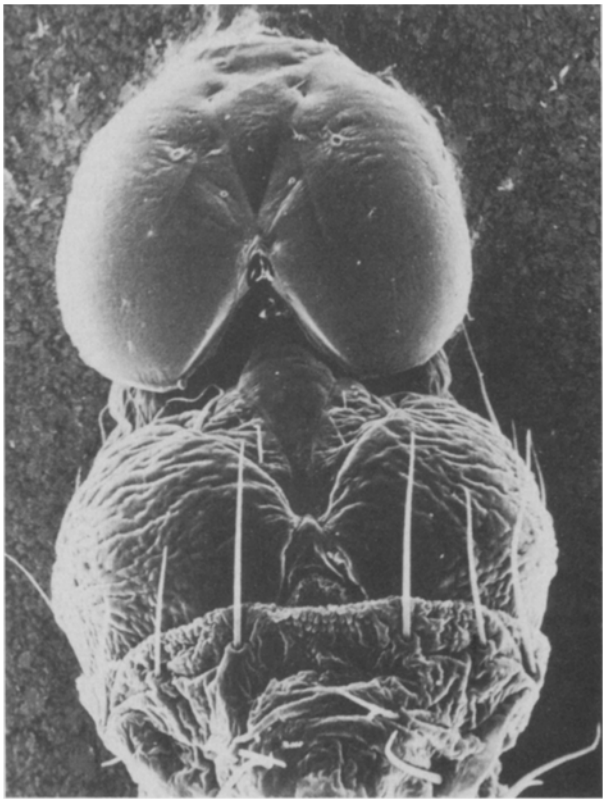

Figure 2. Electron-micrograph (scanning) of *A. assectella* larva killed after feeding with digitonin in the diet: anterior part with two head capsules and incompletely outcasted exuviae.

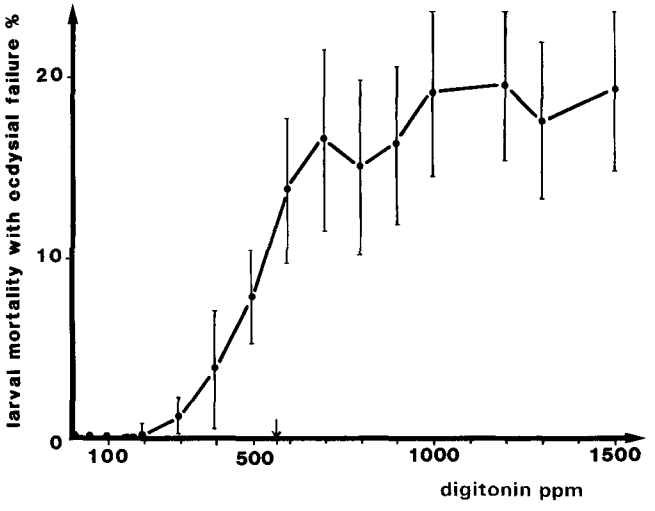

Figure 3. Mortality with ecdysial failure (mainly at the 2nd. molting) of *A. assectella* larvae reared in diets with increasing concentrations of digitonin, in the experiment illustrated in figure 1.

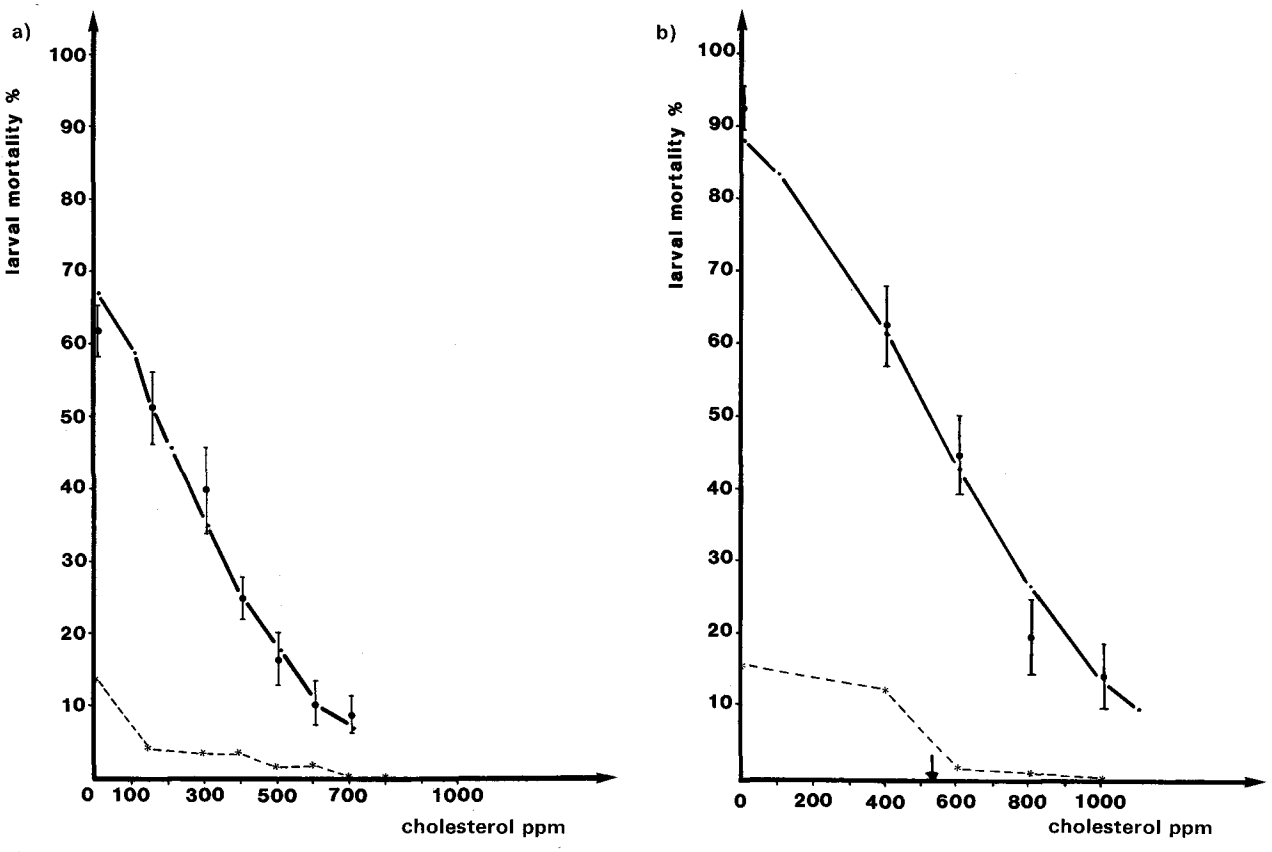

Figure 4. Decreasing mortality of *A. assectella* larvae, with additions of cholesterol to digitonin-containing diet. Constant concentrations of digitonin: 600ppm (fig. 4a) or 800ppm (fig. 4b). Total mortality and adjustment to normal distribution (points); mortality with ecdysial failure (asteriks); arrow indicates DL 50.

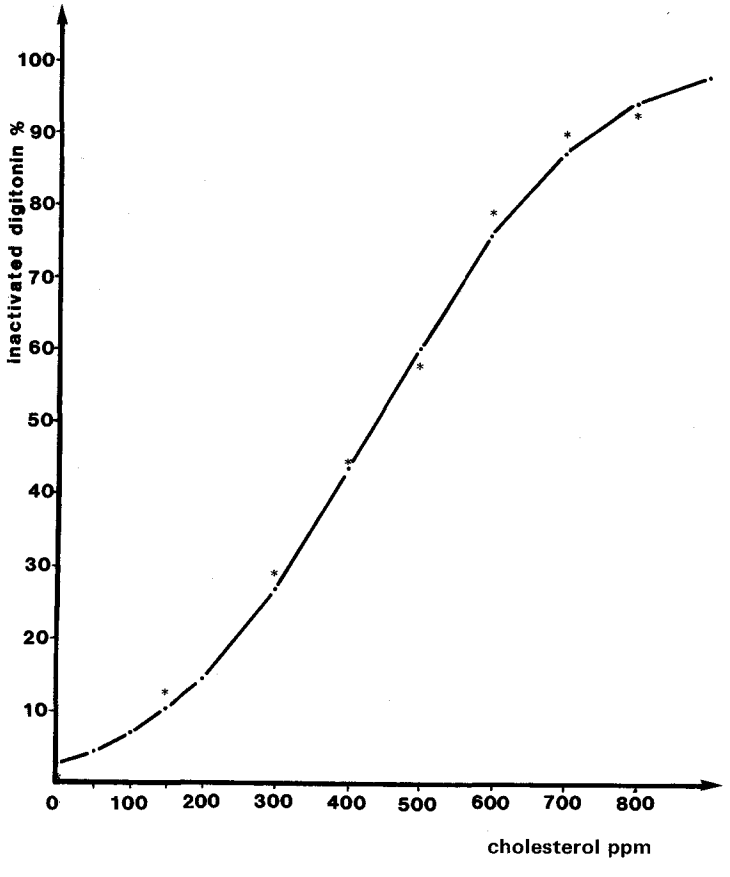

Figure 5. Inhibition of toxic activity of digitonin by increasing amounts of cholesterol added to the diet of *A. assectella*, calculated from data given in figure 1 and figure 4a.

mortality was observed during pupal molting or adult emergence.

More precise data on the critical development stage were obtained by experiments in which larvae were transferred at a known developmental stage (table 2): larvae reared on a standard diet were collected at different instars to be fed with 1000 ppm digitonin-supplemented diet. 30 larvae of each instar were taken for each transfer, and dead larvae appeared only 5 days after transfer. Efficiency of digitonin decreased with age of larvae; effect of ingestion was delayed until the second molting after transfer.

Inhibition of digitonin effects by cholesterol. Experiments were performed with two concentrations of digitonin in the diet. Chosen concentrations (600 ppm and 800 ppm) exceeded LD50. Several quantities of cholesterol were added to the diet; total mortality and mortality with ecdysial failure were determined (fig. 4a, b). Mortality regularly decreased as increasing amounts of cholesterol were added; it was identical with control (diet with only cholesterol) at 800 ppm (fig. 4a) or 1000 ppm (fig. 4b) of cholesterol added, whereas symptomes of ecdysial failure almost completely disappeared.

To evaluate the rate of digitonin inactivation by cholesterol, the data in figures 1 and 4 were used. They establish the relationship between cholesterol concentration and the remaining biological activity of the digitonin. Figure 5 shows the curve of 'neutralization'; the rate calculated by this method was about 5 moles of cholesterol for 1 mole of digitonin. Restoration effect of cholesterol also depended on larval instar. It has been observed after transferring larvae from a digitonin-containing diet (500 ppm) to a cholesterol-containing one (1000 ppm); larvae survived normally when they were taken before the third instar.

Discussion and conclusion. Inhibition of molting has been observed after feeding with several kinds of compounds[5-7]. Such effects have been observed when leek moths were reared on leek flowers. In leek flowers, the active compound was identified as a steroidal saponin in which the nature of the aglycone is close to that of digitonin. Our experiments show that digitonin produced the same effects as leek flowers.

Properties of saponins have been described in several reviews[8]. Saponins are very powerful hemolytic substances and emulsifiers which can form complexes with cholesterol. The first two properties seem to be of no interest with regard to insect physiology. In the previous data on saponin effects on insects (*Melolontha melolontha* or *Tribolium castaneum*) the authors have only reported inhibition of growth and acute toxicity with death of fed animals[9,10].

In *A. assectella*, precise observations of leek-flower saponin or digitonin effects indicated that a cause of mortality was the inhibition of ecdysis; the affected larvae remained enclosed within the old cuticle. Since adding cholesterol to the diet medium abolishes the effects, we considered the ability to form a complex as a hypothesis accounting for ecdysial failure.

It is well known that insects are unable to synthesize cholesterol, in spite of the fact that it is absolutely necessary for their metabolism[11] and for the biosynthesis of the molting hormones. It can be hypothesized that digitonin or leek-flower saponin acts by reducing the cholesterol level in larvae. In rats[12] or monkeys[13] saponins were reported to prevent intestinal absorption and increase the fecal excretion of neutral steroids. Cholesterol added to the diet abolishes the effects; results similar to those obtained with *A. assectella*. The question then becomes; what can be the link between the observed effect and a lowering of cholesterolemia? As cholesterol is a precursor of ecdysteroids the saponins could cause a reduced amount of the cholesterol to be available for ecdysteroidal hormone synthesis. As long as the hemolymph cholesterol titer has a low value, we can obtain complete complex formation, then a total inhibition of ecdysteroid synthesis; this might occur primarily during the first and second larval instars.

Correlations between inhibition of ecdysis and decrease of hemolymph ecdysteroid titre have been described[14] after treatment of the fifth larval instar of *Oncopeltus fasciatus* with 2-acetylpyridine thiosemicarbazones. Though ecdysteroids also have a 3β-OH, the complex apparently could not be directly obtained with the hormone. An antagonistic effect with ecdysteroids would be revealed by ecdysial symptoms independently of the larval instar treated. Moreover, ecdysteroids added to the digitonin-containing diet produced additional characteristic mortality, suggesting that the two products do not act at the same physiological level.

Ecdysis inhibition after digitonin treatment in *A. assectella* revealed similar pathophysiological symptoms to those described by Kubo et al.[15] after feeding either a methanol extract of *Plumbago capensis* roots or purified 'plumbagin'. With this ecdysis inhibitor, the described symptoms were also obtained during the first and the second larval instar. Kubo et al.[15] demonstrated the plumbagin inhibits chitin synthetase activity to the same extent as polyoxin D, a well known chitin synthesis inhibitor isolated from *Streptomyces cacoi* var. *asoensis*[16]. This means that ecdysis inhibition can also be produced by several integumental defects. Digitonin action seems very complex and its mode of action in causing ecdysial failure is not clear. However, investigating this compound is of interest for a better understanding of the epidermal cell activity, a specific target for the discovery of new insect growth regulators. *Acrolepiopsis assectella* does not appear to be the most suitable biological material for complementary experiments, because of the small size of the animals and the fact that they live inside the medium.

* Acknowledgment. We thank Mme Annie Heinzmann for technical assistance.

1 Arnault, C., Ent. exp. appl. *25* (1979) 64.
2 Dr J. Harmatha., Institute of Organic Chemistry of Biochemistry, Czechoslovak Academy of Sciences, 16610 Praha 6, Czechoslovakia.
3 Malinow, M.R., McLaughlin, P., Kohler, G.O., and Levingston, A.L., Steroids *29* (1977) 105.
4 Malinow, M.R., McLaughlin, P., and Stafford, C., Am. J. clin. Nutr. *31* (1978) 814.
5 Kubo, I., and Klocke, J.A., Plant Resistance to Insects, ACS Symposium series, Washington, p. 329 (1983).
6 Kubo, I., Klocke, J.A., and Asano, S., Agric. biol. Chem. *45* (1981) 1925.
7 Kubo, I., Klocke, J.A., and Asano, S., J. Insect Physiol. *29* (1983) 307.
8 Mahato, S.B., Ganguly, A.N., and Sahu, N.P., Phytochemistry *21* (1982) 959.
9 Horber, E., in: Insect and Mite Nutrition, p. 611. Ed. J.G. Rodriguez. Amsterdam 1972.
10 Shany, S., Birk, Y., Gestetner, B., and Bondi, A., J. Sci. Fd Agric. *21* (1970) 131.
11 House, H.L., in: The Physiology of Insecta, vol. 1. Ed. M. Rockstein. Academic Press, New York 1974.
12 Malinow, M.R., McLaughlin, P., Papworth, L., Stattford, C., Kohler, G.O., Levingston, A.L., and Cheeke, P.R., Am. J. clin. Nutr. *30* (1977) 2061.
13 Malinow, M.R., Connor, W.E., McLaughlin, P., Stafford, C., Lin, D.S., Levingston, A.L., Kohler, G.O., and McNulty, W.P., J. clin. Invest. *67* (1981) 156.
14 14 Kelly, Y.L., Redfern, R.E., DeMilo, A.B., and Borkovec, A.B., Pestic. Biochem. Physiol. *17* (1982) 35.
15 Kubo, I., Uchida, M., and Klocke, J.A., Agric. biol. Chem. *47* (1983) 911.
16 Endo, H., Kakiki, K., and Misato, T., J. Bact. *114* (1971) 189.

0014-4754/85/081074-04$1.50 + 0.20/0