Evidence for a Cardenolide Carrier in *Oncopeltus fasciatus* (Dallas) (Insecta: Hemiptera)

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Oncopeltus fasciatus (Hemiptera) is morphologically and physiologically adapted to sequester cardiac glycosides for its chemical defence against predators. As a prerequisite for cardenolide utilization this bug has to take up and store the dietary cardenolides. Using isolated midguts it could be shown experimentally that the resorption of [^{3}H]digoxin is time-, and temperature- dependent. The hydrogen ion concentration for uptake is optimal between pH 5.5 and 7 and an activation energy of 45 to 49 kJ/mol can be derived from an Arrhenius plot. Uptake can be inhibited competitively by the polar cardiac glycoside convallatoxin. Sulfhydryl group reagents (N-ethylmaleimide), membrane destabilizing compounds and respiratory chain inhibitors reduce digoxin resorption, as do inhibitors of sugar transport (e.g., phloridzin). The experimental data thus provide evidence for a cardenolide carrier in midgut cells of *O. fasciatus.*

Introduction

Plants produce a wide variety of secondary metabolites, such as alkaloids, terpenoids and glycosides, many of which serve as antiherbivoral or antimicrobial defence compounds (Wink, 1988; Harborne, 1993; Bernays and Chapman, 1994). However, a substantial number of mono- or oligophagous insect species have evolved which are morphologically and physiologically adapted to exploit the particular defence chemistry of their hostplants (Duffey, 1980; Harborne, 1993; Bernays and Chapman, 1994)

Larvae of the milkweed bug, *Oncopeltus fasciatus* (Dallas) (Hemiptera) feed on milkweed plants (genus *Asclepias*) which produce cardiac glycosides (CG) for chemical defence against herbivores. Pioneering studies by G.G.E.Scudder, S.S. Duffey, and M.B.Isman have shown that *O. fasciatus* is specialized for uptake, sequestration and tolerance of cardiac glycosides, similar to a number of other aposematically coloured Lepidoptera, Hemiptera and Coleoptera (Feir and Suen, 1971; Duffey and Scudder, 1972, 1974; Isman *et al.,* 1977; Duffey *et al.,* 1978; Vaughan, 1979; Duffey, 1980; Scudder and Meredith, 1982a,b; Scudder *et al.,* 1986; Moore and Scudder, 1985, 1986; Malcolm, 1990). It could be shown experimentally that cardenolide-rich milkweed bugs may become distasteful for predators (Scudder *et al.,* 1986) similar to the situation in cardenolide-storing monarch butterflies (Reichstein *et al.,* 1968; Brower *et al.,* 1975, 1982; Seiber *et al.,* 1980, 1986; Fink and Brower, 1981; Brower, 1984; Brower and Fink, 1985; Cohen, 1985; Ritland, 1991).

A requirement for an efficient uptake and storage of cardenolides would be a selective carrier system in midgut epithelia and in the storage compartments. In larvae of *Syntomeida epilais* and *Danaus plexippus* which also store CGs for chemical defence we had obtained evidence that CGs are resorbed from midguts by a carrier-mediated process (Nickisch-Rosenegk et al. 1990a; Frick and Wink, 1995). In analogy, we analyzed in this study whether digoxin is taken up by midguts of *O. fasciatus* larvae by a similar mechanism. Since uptake and carrier experiments are difficult to carry out with intact larvae, midguts were isolated from L_5 larvae and used for *in vitro* uptake studies.

Material and Methods

Animals

Oncopeltus fasciatus was reared in our laboratory on sunflower seeds at 23° C.

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Carrier studies

L5 larvae were dissected in a modified Ringer solution (pH 6). Midguts were excised and kept in an incubation buffer containing 5.5 mM glucose, 2.6 mM KCl, 13.7 mM NaCl and either 50 mM MES (for pH 4 to 7) or 50 mM Tricine (for pH 8 to 9) for 20 to 40 min at room temperature.

Usually 9 midguts of equal size were added to 100 µl incubation buffer and reactions were started with 100.000 cpm [^{3}H]digoxin (specific activity 976 GBq/mmol; NEN). Except for kinetic experiments, assays were run at 25 °C for 60 min. Then the midguts were washed twice with 0.2 ml cardenolide-free incubation buffer and three midguts each were transferred into an Eppendorf vial containing 100 µl 2 M HCl. Following storage at −80 °C midguts were homogenized with a ultrasonic disintegrator. The radioactivity of the homogenates (as a measure for the amount of [^{3}H]digoxin taken up) was measured in a scintillation counter (counting efficiency 65%). In addition, the radioactivity of the residual incubation buffer was measured for the calculation of the percentage of radioactivity taken up by the midguts. As a control, midguts were incubated in the above-mentioned buffer to which 1 µCi tritiated water had been added and were processed in the same way as in the [^{3}H]digoxin experiments. All sets of experiments were repeated at least twice. Data given are means ± standard error of the mean.

Results

Midguts from L_5 larvae were incubated with [^{3}H]digoxin for differing time intervals and [^{3}H]digoxin uptake was monitored by measuring the radioactivity of the midgut cells. As can be seen from Fig. 1, uptake of [^{3}H]digoxin is almost linear with time for the first 60 min. Some [^{3}H]digoxin seems to be quickly adsorbed to the cell membranes, since even the intial "relative uptake values" are about 1% (Figs 1–3). [^{3}H]H_20 which freely diffuses across membranes and equilibrates rapidly between cells and incubation medium, is taken up by 0.6% already after 5 min and does increase further whereas [^{3}H]digoxin becomes enriched in the midgut epithelia reaching 4.2% as a maximum after 60 min. These data indicate that [^{3}H]digoxin accumulates against a concentration gradient. Also the polar [^{3}H]ouabain is resorbed by *O. fasciatus* midguts but the rates are much lower, reaching a relative uptake of 0.23 ± 0.03% after 90 min (Fig.1).

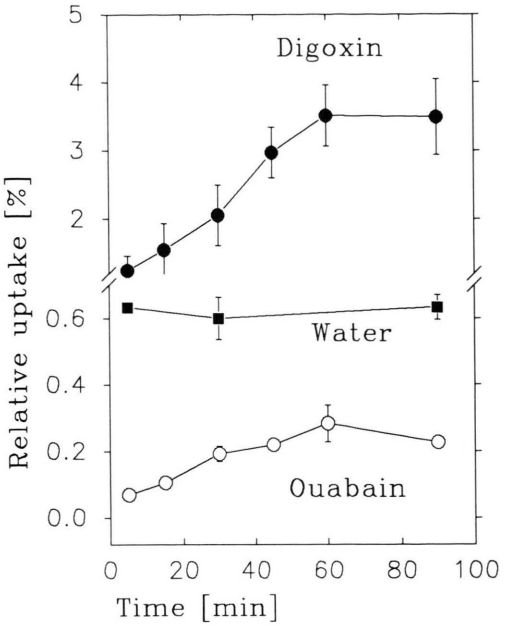

Fig. 1. Time course for the uptake of [^{3}H]digoxin, [^{3}H]-ouabain and [^{3}H]H_2O by isolated midguts of *Oncopeltus fasciatus*. The total radioactivity present in the incubation buffer at the beginning of the experiment was set 100%. Reactions were carried out at pH 7 and 23 °C. Values represent means ± s.e.(n = 3).

The following experiments were carried out to determine the effect of temperature and hydrogen ion concentrations. As can be seen from Fig. 2, uptake is optimal between pH 5.5 and 6.5 and displays a clear temperature-dependence. An activation energy of 49.4 kJ/mol (pH 6) or 45.4 kJ/mol (pH 7) can be derived from an Arrhenius plot (Fig. 3). [^{3}H]Digoxin uptake can be inhibited by the polar convallatoxin (probably through substrate competition) but not by ouabain (Fig. 4) suggesting substrate specificity.

The effect of several modulators/inhibitors on [^{3}H]digoxin uptake is shown in Table I: Glucose could be replaced by mannose and mannitol. Both K^+ and Na^+ were needed for optimal activity. [^{3}H]digoxin uptake was inhibited by compounds which bind to sulfhydryl groups of proteins, such as N-ethylmaleimide and to a lesser degree *p*-hydroxymercuribenzoate. Mercaptoethanol had no effect, suggesting that S-S-bonds have no role

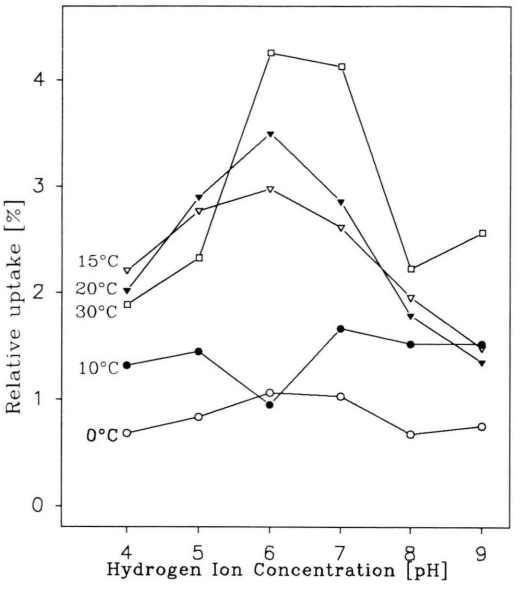

Fig. 2. Influence of hydrogen ion concentration and temperature on the uptake of [^{3}H]digoxin by midguts of *O. fasciatus*. Reactions were terminated after 50 min.

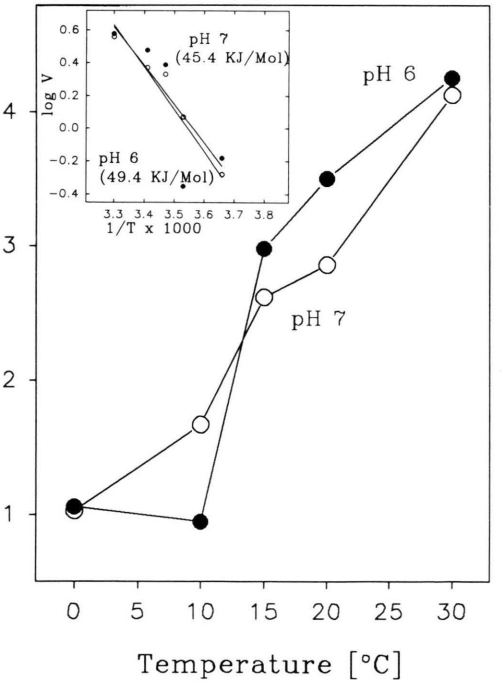

Fig. 3. Determination of activation energy for [^{3}H]-digoxin uptake (pH 6 and pH 7). The insert illustrates a corresponding Arrhenius plot.

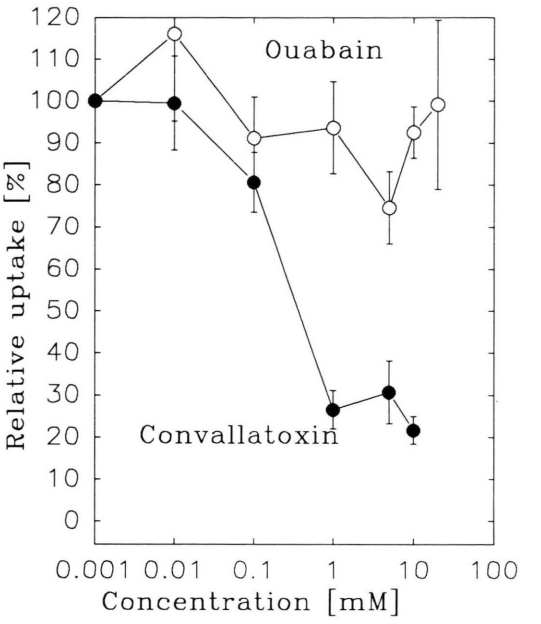

Fig.4. Inhibition of the uptake of [^{3}H]digoxin by convallatoxin or ouabain (final concentrations between 0.01 and 20 mM). Reactions were performed at 23 °C for 50 min both at pH 7; control = 100%. Values represent means ± s.e.(n = 6).

since this compound reduces S-S bonds to free SH groups. KCN and 2,4-dinitrophenol (1 mM each) which block the mitochondrial respiratory chain inhibited [^{3}H]digoxin uptake by 47% and 31%, respectively.

Membrane integrity appears to be important for uptake, since detergents (saponin, digitonin, triton X-100, tween) inhibited the process. A tissue denaturation by 1 M HCl had a similar effect. CCCP destroys proton gradients at biomembranes; only at relatively high concentrations of 1 mM an uptake inhibition of 27% was observed.

Phloridzin (1 mM) inhibits sugar transport by 49%; 0.5 mM cytochalasin B, an inhibitor of fluid phase encytosis is also active, but not colchicine which binds to tubulin.

Discussion

If the cardenolide profiles, present in the host plants, are compared with those in the milkweed bug, it is apparent that they do not match in both qualitative and quantitative terms (Vaughan, 1979; Feir and Suen, 1971; Duffey and Scudder, 1974; Isman *et al.*, 1977; Duffey *et al.*, 1978; Scudder and

Table I. Modulation of [^{3}H]digoxin uptake in midguts of *Oncopeltus fasciatus*. Midguts were added to the incubation buffer plus inhibitors; 10 min. later, the reaction was started by adding [^{3}H]digoxin.

Compound	Relative uptake activity $\bar{x} \pm$ s.e. (n) (control = 100%)
1. Sugars	
Substitution of glucose by	
Mannose (5.5 mM)	117.4 ± 12.9 (6)
Mannitol (5.5 mM)	105.7 ± 16.9 (6)
2. Salts	
No NaCl; only KCl (13.7 mM)	74.1 ± 8.4 (6)
No KCl; only NaCl (13.7 mM)	68.9 ± 11.1 (6)
3. ATP	
plus MgATP (10 mM)	82.6 ± 11.7 (12)
plus MgATP (5 mM)	99.2 ± 10.9 (12)
4. Inhibitors	
a) SH reagents	
Mercaptoethanol (10 mM)	96.2 ± 11.3 (6)
N-Ethylmaleimide (1 mM)	28.9 ± 5.7 (9)
(0.75 mM)	63.1 ± 7.9 (12)
(0.5 mM)	87.3 ± 8.8 (12)
p-Hydroxymercuribenzoate (1 mM)	60.1 ± 6.2 (5)
b) inhibitors of respiratory chain	
2,4-Dinitrophenol (1 mM)	69.1 ± 7.6 (5)
KCN (1 mM)	53.4 ± 3.7 (6)
(0.5 mM)	64.3 ± 8.1 (6)
c) Effects on membranes	
CCCP* (1 mM)	72.6 ± 7.3 (6)
(0.5 mM)	89.7 ± 10.9 (6)
Digitonin (1 mM)	37.3 ± 16.3 (6)
Saponin (0.1 mM)	27.7 ± 7.2 (5)
Triton X100 (0.2%)	68.1 ± 11.6 (6)
Tween (0.2%)	70.2 ± 7.9 (4)
Dimethylsulfoxide (1%)	69.2 ± 4.5 (6)
HCl (1 M)	36.7 ± 6.6 (7)
d) Transport inhibitors	
Phloridzin (1 mM)	50.6 ± 7.2 (9)
(0.5 mM)	91.9 ± 11.1 (10)
Colchicine (1 mM)	75.3 ± 21.6 (6)
Cytochalasin B (0.5 mM)	61.9 ± 5.3 (6)

* CCCP, carbonylcyanide-*m*-chlorophenylhydrazine.

Meredith, 1982a; Moore and Scudder, 1985; Scudder *et al.*, 1986). A similar picture emerged for other CG storing insects, such as *Danaus plexippus, D. gilippus, D. chrysippus* (Reichstein *et al.*, 1968; Roeske *et al.*, 1976; Seiber *et al.*, 1980, 1986; Lynch and Martin, 1987; Martin and Lynch, 1988; Malcolm and Brower, 1989; Malcolm *et al.*, 1989; Malcolm, 1990), *Caenocoris nerii*, *Spilostethus pandurus* (v. Euw *et al.*, 1971), *Poikilocerus bufonius* (v. Euw *et al.*, 1967), and *Aphis nerii* (Rothschild *et al.*, 1970; Malcolm, 1990).

Selective sequestration with aid of a cardenolide carrier?

It has been shown that *Danaus* and *Oncopeltus* are able to metabolize some of the dietary cardenolides (Scudder and Meredith, 1982b; Brower, 1984; Scudder *et al.*, 1986). For example, a cardenolide may be hydrolyzed to obtain the corresponding aglycone (Seiber *et al.*, 1980). But metabolism alone can only partly explain the difference of cardenolide profiles between host plants and insects. When feeding cardenolides to moth larvae (*Syntomis mogadorensis* and *Creatonotos transiens*) which do not encounter cardenolides in their diet, we found that the glycosides were not taken up but eliminated with the faeces (Wink and Schneider, 1990). Also the midgut of *Schistocerca gregaria* and *Periplaneta america* was impermeable to ouabain and other cardenolides (Scudder and Meredith, 1982b). These data implicate that midgut membranes of insects function as a permeability barrier (at least for the polar compounds) and that a special mechanism needs to be present in insects for resorption and storage of cardiac glycosides.

Isolated midguts of *O. fasciatus* take up [^{3}H]digoxin in a time-dependent way influenced by hydrogen ion concentration of the incubation medium (Figs 1–2). The hydrogen ion concentration was optimal between pH 5.5 and 7 which is in the range of carrier-systems for secondary metabolites in other insects: uptake of pyrrolizidine alkaloid N-oxides in *Creatonotos transiens*: pH 5 (Wink and Schneider, 1988); uptake of 13-hydroxylupanine in *Uresiphita reversalis*: pH 9 (Wink *et al.*, 1991); and uptake of digoxin in *Syntomeida epilais*: pH 4 (v. Nickisch-Rosenegk *et al.*, 1990a) and of ouabain in *D. plexippus*: pH 6 (Frick and Wink, 1995). The hydrogen ion concentration in the midgut of insects is in the range between pH 2.6 and 9.0 (Appel and Martin, 1990). Since pH optima of carriers vary between pH 4 and pH 9, it is not clear whether hydrogen concentrations in the gut corre-

late with pH optima determined for the carrier systems.

[^{3}H]Digoxin uptake in *Oncopeltus* is temperature-dependent and displays a maximum at ≥ 30 °C, whereas the optimum was 24 °C in *Creatonotos, Syntomeida, Danaus,* and *Uresiphita* (Fig.2). The data of temperature dependence can be converted in an Arrhenius plot to calculate the corresponding activation energy. For pH 6 and 7 we obtained an activation energy of 45 to 49 kJ/mol (Fig.3) which indicates that the uptake is a catalyzed process (carrier-free diffusion shows values below 20 kJ/mol). Similar values were determined in other carrier systems: *C. transiens* 55–81 kJ/mol (Wink and Schneider, 1988), *S. epilais* 59 kJ/mol (v.Nickisch-Rosenegk *et al.,* 1990a), *D. plexippus* 49 kJ/mol (Frick and Wink, 1995), and *U. reversalis* 55–78 kJ/mol (Wink *et al.,* 1991).

Furthermore, the uptake of [^{3}H]digoxin was inhibited by adding another cardenolide, i.e. the polar convallatoxin, to the incubation mixture (Fig.4) whereas ouabain had no significant effect suggesting the presence of a cardenolide carrier with substrate specificity. A simple diffusion process would not be influenced by such an addition.

The experiments with modulators/inhibitors provide first clues to the underlying mechanism. [^{3}H]digoxin can only accumulate in midgut epithelia if membrane stability is preserved: 1 M HCl, 0.1 mM saponin and detergents strongly inhibit the process. The SH-reagent data imply that free sulfhydryl groups but not S-S-bonds are important for transport activity. The reduction of [^{3}H]digoxin uptake by phloridzin indicates that the cardenolide carrier might share some properties with glucose transporters or may be derived from them. Since cardenolides are glycosides, such a correlation would be plausible. If the cytochalasin B effect was specific, then a participation of fluid phase endocytosis should be additionally considered.

Yoder *et al.* (1976) had used a similar experimental approach with isolated midguts. They also found a reduced ouabain uptake as compared to that of digitoxin. Digitoxin uptake was linear with time and stimulated at a Na^+ concentration of 154 mM. Since they could not determine a substrate saturation (we obtained the same result in preliminary experiments; data not shown), they explain their results as a diffusion. Duffey *et al.,* (1978) and Scudder *et al.,* (1986) reached a similar conclusion because they found no saturation kinetics in their *in vivo* experiments. We suggest that these earlier experiments can be explained in terms of a facilitated diffusion, which excludes an active transport but not a carrier-mechanism. In addition, the lack of saturation kinetics imply that a possible diffusion component is involved in the resorption of lipophilic CGs. Another evidence for free diffusion derives from our inhibitor experiments: Since the inhibitor experiments never achieved a 100% reduction (the lowest level was 27% for saponin; Table I) we assume that the residual "uptake" was due to simple diffusion and to partitioning of the cardenolide in the membrane fraction.

In conclusion, the data shown in Figs. 1–4 and Table I suggest that digoxin uptake can be attributed to both, a carrier-mediated transport and a simple diffusion. The experiment with tritiated water suggests that [^{3}H]digoxin was concentrated in the midgut epithelia against a concentration gradient (Fig.1). This would require an energy dependent uphill transport. Since KCN and DNP, which are inhibitors of endogenous respiratory chain and thus of ATP synthesis, also reduce digoxin uptake, an indirect coupling of cardenolide uptake to ATP hydrolysis would be a possibility. MgATP was inactive in our experiments (Table I). However, it is likely that MgATP does not reach the cytoplasm of epithelial cells and therefore cannot fuel the transport process.

Adaptations of Oncopeltus towards cardenolide utilization

Oncopeltus represents a herbivore which is physiologically and morphologically adapted to take up, sequester and utilize cardenolides. As a first step a carrier-mediated cardenolide uptake is active besides a free diffusion for the lipophilic glycosides. Such a carrier system would provide a means to concentrate cardenolides from plants whose cardenolide concentrations are low. In addition, a carrier would discriminate cardiac glycosides already in the midgut lumen and to discharge all those compounds which do not bind to the carrier, with the faeces (except for the lipophilic compounds which are partly resorbed by free diffusion).

After resorption of cardenolides by the midgut epithelia these compounds are released into the hemolymph from which they are readily cleared and deposited in the integument (Scudder *et al.,* 1986). A remarkable morphological adaptation towards CG storage was observed: The milkweed bug has a modified integument which is composed of a double-layered epidermis with an inner layer (the dorsolateral space) which constitutes the cardenolide storage compartment. On both sides of thorax and abdomen thin cuticle areas exist which rupture when the bug is attacked. As a result the cardenolides are released from the dorsoventral space and the predator is immediately confronted with the bitter cardenolides (Scudder and Meredith, 1982a; Scudder *et al.,* 1986). It is remarkable that plant-derived toxins are often deposited in the integument or cuticles: other examples are: pyrrolizidine alkaloids in arctiid moths (Egelhaaf *et al.,* 1990; v.Nickisch-Rosenegk *et al.,* 1990b) and ithomiine butterflies (Brown 1984); cardenolides in *Danaus* (Brower and Glazier, 1975; Brower *et al.,* 1988; Frick and Wink, 1995), *Syntomeida* (v. Nickisch-Rosenegk *et al.,* 1990a); cyanogenic glycosides in *Zygaena* (Franzl *et al.,* 1988), phenols in tree locusts, *Anacridium melanorhodon* (Bernays and Woodhead, 1982), cannabinol in *Arctia caja* (Rothschild *et al.,* 1977), and quinolizidine alkaloids in *Uresiphita reversalis* (Wink *et al.,* 1991).

Since cardiac glycosides inhibit Na^+/K^+-ATPase in insects and other animals, *Oncopeltus* must have evolved insensitivity at this molecular target or other detoxification mechanisms (such as storage in the integument). It was calculated that *Oncopeltus* larvae can tolerate 1954-fold and 7288-fold, respectively, the LD_{50} ouabain dose of the CG-sensitive insects, *Schistocerca gregaria* and *Periplaneta americana* (which would kill almost any other animal) and its Na^+/K^+-ATPase cannot be inhibited by cardenolides (Moore and Scudder, 1986) similar to the situation in the monarch butterfly (Vaughan and Jungreis, 1977; Holzinger *et al.,* 1992). At the molecular level we found that in monarch butterflies one amino acid is different in the putative ouabain binding site (length 12 amino acids) of Na^+/K^+-ATPase and we have suggested that ouabain can no longer bind and thus insensitivity is achieved (Holzinger *et al.,* 1992). A corresponding study concerning *Oncopeltus fasciatus* is in progress in our laboratory (F.Holzinger and M. Wink, in preparation). Although the picture is certainly far from complete it is nevertheless evident that a series of complex gene-encoded adaptations are necessary in a herbivore before it can utilize a dietary defence compound for its own protection.

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