CYANOGENESIS AND THE FEEDING PREFERENCE OF ACRAEA HORTA (L.) (LEPIDOPTERA: ACRAEINAE)

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INTRODUCTION AND HISTORICAL PERSPECTIVES

The statement that similar sorts of plants often have similar medicinal properties (i.e. contain similar chemicals) is at least 300 years old, while the concept probably dates back several thousand years (Stace, 1980). Indeed, a knowledge of the secondary chemicals produced by different plant species has played an important role since the early stages of man's cultural, and probably evolutionary, development (e.g. Leopold and Ardrey, 1972). Until recently, however, the biological role of this large group of compounds has remained largely obscure, with many plant physiologists regarding them as waste products and of no possible survival value to plants (Harborne, 1982). In recent years a tremendous increase in attention paid to these compounds has led to the suggestion that plant secondary substances evolved as herbivore deterrents, and diversified with the plants producing them as herbivores evolved various means of coping with their toxicity. In what follows I present an overview of events leading to the establishment of this theory, and a history of the discovery that some insect herbivores have turned the toxicity of plant secondary compounds to their own advantage, thereby becoming exclusively associated with toxic food plants. It will be seen that although this theory of plant-herbivore coevolution has become generally accepted, it has yet to be tested for a wide range of herbivores and chemical types. From this view I have taken a detailed look at a specific group of herbivores and plant toxins: Lepidoptera feeding on cyanide-producing plants.

Stahl (1888) was the first to suggest that secondary plant chemicals may play a direct role in the feeding behaviour of herbivorous animals and this idea was successfully put to the test by Verschaffelt (1910) in his investigation of *Pieris* butterflies and their cruciferous foodplants. In subsequent years, as further evidence amassed, this theory was restated from various perspectives (e.g. Painter, 1936, 1951; Dethier, 1941, 1947, 1954; Fraenkel, 1953). It was not, however, until Fraenkel (1959) that a comprehensive statement regarding the role of insects in the evolution of secondary plant compounds appeared. In this now classic paper Fraenkel suggested that the basic dietary requirements of all herbivorous insects are similar, and that these can be met by feeding on the tissues of any green plant. The observed feeding patterns of herbivorous insects - which never feed indiscriminately on all plants available to them - must therefore be due to the presence of secondary plant compounds. These "...serve as repellants to insects (and other animals) in general and as attractants to those few which feed on each plant

species." (Fraenkel, 1959 p.1466). This view was subsequently criticised, particularly on the strength of evidence suggesting that (a) plants differ in the balance (i.e. proportions) of nutritionally important substances they contain, (b) different insect species have subtle differences in nutritional requirements which are largely based on nutrient balance, and (c) insects are capable of selecting food according to its suitability as a means of satisfying these specific dietary requirements (e.g. Thorsteinson, 1960; House, 1961 *et seq.*; Beck, 1965; Kennedy, 1965). In 1969 Fraenkel published a revised version of his theory, intended chiefly as a rejoinder to his critics, in which he conceded that his original statement was too extreme in its formulation, but he nevertheless re-emphasised the primacy of secondary chemicals in insect host selection.

In this way these chemicals came to be known as 'secondary' compounds, as they were considered to have an ecological function and to play no role in the primary metabolism of plants. This terminology has, however, been criticised on the grounds that at least some of these substances are believed to have originated as important compounds of plant primary metabolism (Seigler and Price, 1976; Beck and Reese, 1976; Jones, 1979). Various alternative terms have been proposed, including 'allelochemics' (Whittaker, 1970; Whittaker and Feeny, 1971), 'allelochemicals' (Slansky, 1982; Rosenthal, 1986) and 'ecological chemicals', the study of which has been termed 'ecological chemistry' (Brower and Brower, 1964; Brower, 1969; Rosenthal, 1986). Despite these various protestations, the terminology has not been standardized and all these terms are still in use. For the purposes of this thesis I use the term 'secondary compound', since I consider 'secondary' to refer to the present function of the compound rather than the function for which it may have originally evolved. Thus, a substance may have evolved in plants as a product of primary metabolism, but its subsequent elaboration for the secondary function of defence would earn it the appellation 'secondary compound'.

In the 1960's the seminal ideas of Fraenkel and others were further developed, when Ehrlich and Raven (1965) proposed the theory of coevolution between plants and insects to explain the distinct patterns of host choice by Lepidoptera. Briefly stated, this theory suggests that a mutation for the production of chemicals, toxic or otherwise offensive to herbivores by plants, may carry a plant population into a new adaptive zone where it is free to diversify because of its protection from herbivorous animals. Insects, however, can evolve in response to physiological obstacles and an appropriate mutation could in turn carry an insect population into

an adaptive zone where it is free to diversify in the absence of other, susceptible, feeders. In this way both the plants and the insects have become diverse groups, and a tremendous diversity in the chemical structures of secondary plant compounds has developed. This theory adequately explains the fact that related insects often feed on related plants, or on plants containing similar secondary chemicals (Harborne, 1982).

Although this theory of plant-insect coevolution has now been generally accepted (e.g. Schoonhoven, 1972a; Feeny, 1976; Harborne, 1982), there is a lack of supportive evidence from empirical investigations into the patterns of plant, insect and secondary chemical diversity and their causes (Smiley, 1985). This lack of evidence may, in part at least, be due to experimental difficulties encountered in attempts to demonstrate a defensive function for plant secondary compounds (Jones, 1971; Feeny, 1975). There is, nonetheless, a notable exception to be found in the work of Berenbaum (1983), which provides empirical evidence supporting the coevolutionary theory. Results of Berenbaum's study suggest that the evolution of the ability to produce toxic furancoumarins created a herbivore-free adaptive zone favouring speciation in umbelliferous plants, and that papilionid butterflies, having adapted to cope with these toxins, were in turn free to diversify in the absence of competitors. There exists, on the other hand, evidence from a recent study of *Heliconius* butterflies and their *Passiflora* food plants which forcibly challenges the pervasiveness of the coevolutionary theory, suggesting that other (e.g. ecological) factors may play a primary role in determining patterns of insect herbivory (Smiley, 1978, 1985).

It thus appears that, although secondary plant compounds have been established as an important factor in the evolution of insects and their host plants, other less-explored factors also play a role. What therefore remains to be answered is not whether, but *to what extent* the coevolutionary theory can be invoked to explain the patterns of diversity in insects, plants and plant secondary compounds we observe in nature. Any satisfactory answer to this question must await further studies on the patterns of host utilisation for a wide range of insects, host plants and plant secondary compounds. Whatever the outcome of this question, it nevertheless remains true that the articulation of the coevolutionary theory served as an important catalyst in the rapid expansion of the field of chemical ecology (e.g. see Sondheimer and Simeone, 1970; van Emden, 1972; Harborne, 1978; Rosenthal and Janzen, 1979).

THE UTILISATION OF PLANT SECONDARY SUBSTANCES BY INSECTS

A major aspect in the study of chemical ecology is concerned with the response of insects to plant secondary compounds. Insects may either avoid plants producing toxic secondary compounds, or they may excrete (Rothschild, 1972) or detoxify (Frazer and Rothschild, 1961; Hsaio and Fraenkel, 1969; Krieger *et al.*, 1971) such compounds. Some insects have, moreover, evolved the ability to utilise plant secondary substances as a means of recognising their host plants or, through the sequestration and storage of these compounds, in ordering interactions with other organisms around them. This latter category includes their use as insect pheromones (Pliske and Eisner, 1969; Hendry *et al.*, 1975) and, perhaps most interesting from a coevolutionary point of view (Ehrlich and Raven, 1965), as toxins or predator deterrents for use against organisms at higher trophic levels. Whittaker (1970 p.62) introduced the term 'kairomones' for such ".....chemical agents (which are) adaptive to an organism other than their source...." (see also Whittaker and Feeny, 1971).

It was suggested over a hundred years ago that distasteful insects may obtain toxins from the plants they eat (Distant, 1877; Wallace, 1889; Haase, 1896). The first, albeit indirect, evidence of this came from Jones (1937) who showed that beetles eating *Asclepias* plants were rejected by birds and ants, regardless of whether their guts contained remnants of the toxic food plant, or whether they had been evacuated through starvation. It was not, however, until the demonstration by Parsons (1965) that monarch butterflies (*Danaus plexippus* L.) contain cardenolides similar to the digitalis-like toxins produced by their *Asclepias* food plants that definitive evidence began to emerge. That these cardenolides in the monarch are of plant origin was confirmed by the feeding experiments of Brower *et al.* (1967) and the chemical analyses of Reichstein (1967). A parallel study with *Poekilocerus bufonius* (Klug) (Pyrgomorphidae), a north African grasshopper that feeds almost exclusively on *Asclepias* plants, revealed that this species, too, contains cardenolides similar to those in its food plant (von Euw *et al.*, 1967). Specimens reared on non-poisonous plants contained approximately ten times less cardenolide than those reared on the natural food plant, and this provides strong evidence that the toxins are indeed plant-derived. It has subsequently been demonstrated that several other species of grasshoppers, including *Poekilocerus pictus* (Fab.), *Phymateus viridipes* Stal, and *P. bacatus* (Stal) all accumulate the same cardiac glycosides in their tissues as those produced by their food plants (Reichstein *et al.*, 1968).

It has since become increasingly clear that sequestration of plant secondary compounds is a widespread means of defence among herbivorous insects. Interesting circumstantial evidence for this is provided by the observation that virtually all the major groups of defensive chemicals found in arthropods are also represented in plants (Eisner, 1970; Rodriguez and Levin, 1976). There also exists abundant direct evidence. For example, Rothschild (1972) alone lists 43 species from six different orders that are known to sequester secondary chemicals from their food plants, and further examples are listed in Levin (1976) and Duffey (1980). It is immediately clear from this vast body of literature that a wide range of chemical types, plant taxa and insect taxa are implicated in this process. Such remarkable diversity may in itself suggest that no elaborate physiological adaptations are required of insects to accumulate plant toxins in their tissues - a suggestion clearly discussed by Duffey (1980). Further evidence for this comes from the demonstration that certain insects are capable of sequestering plant toxins that they normally never encounter in nature, and, in some cases at least, that these may fortuitously act as potent deterrents to vertebrate predators (Rothschild *et al.*, 1977; Rothschild *et al.*, 1979).

A second function of plant secondary compounds adopted by insects is their use as a means of recognising their host plants. One interesting aspect of this, when considering insects in which there exists a mobile adult phase and a relatively sedentary larval stage (e.g. Lepidoptera), is what the relative discerning capability of both stages should be. Wiklund (1975) has argued that adults should be more selective in where they lay their eggs, since an adult's greater mobility would allow it a second choice were it to alight on a plant of marginal suitability. The larva, on the other hand, should feed on a marginal host if it hatches there since its chances of finding a more suitable host are limited by its lack of mobility. This has been confirmed by Chew (1977) for *Pieris* butterflies. It is nevertheless true that in some cases, depending on particular life cycle characteristics, larval ability to discern potential host plants is important (Dethier, 1954; Ehrlich and Raven, 1965; Fraenkel, 1969; Schoonhoven, 1972b ; Chew, 1977).

There are numerous examples of host recognition by means of secondary compounds, in which just about every class of secondary compound is implicated (e.g. Fraenkel, 1959, 1969; Dethier, 1941, 1954; Schoonhoven, 1968, 1972a; Harborne, 1982). Such recognition may be through the presence of specific recognition factors or through the absence of inhibitory substances in the host plant (examples of both are listed in Schoonhoven, 1972a). A detailed knowledge of

chemosensory events during host recognition has been obtained, for some species at least. Among the most thoroughly studied of these are the large cabbage butterfly (*Pieris brassicae* L.) (Schoonhoven, 1967), larvae of the commercial silkworm (*Bombyx mori L.*) (Ishikawa, 1963) and the tobacco hornworm (*Manduca sexta* Johan) (Schoonhoven and Dethier, 1966). It seems, however, that available knowledge is restricted to sensory (i.e. peripheral) events, and we have little understanding of the central mechanisms involved in the processing of information conveyed to the central nervous system by the chemoreceptors (Dethier, 1972; Dethier, 1980). This is unfortunate since evidence exists to suggest that host discrimination in phytophagous insects is mediated by central mechanisms rather than by specificity in peripheral sensors (Dethier, 1980).

There are also several examples of insects responding to the nutritional content of a plant rather than to plant secondary compounds (e.g. Kennedy and Booth, 1951; Thorsteinson, 1960; Beck, 1965; Kennedy, 1965; House, 1966, 1967, 1969; Dethier, 1972; Schoonhoven, 1972a). This conflicting evidence has given rise to much debate on whether the preferential feeding behaviour of insects is predominantly due to nutritional substances or to the presence of nutritionally unimportant secondary compounds in plants. In addition to the extreme views, various combinations of these have been proposed, assigning different roles to nutrients and secondary compounds in the process of host selection (see Thorsteinson, 1960; Beck, 1965; Kennedy, 1965 for discussions on these). It has also been demonstrated that nutritional substances and secondary compounds may act synergistically in stimulating feeding by insects (see Thorsteinson, 1960 for examples). However, what is sometimes overlooked in this vast literature is that the nutrients *vs* secondary compounds hypotheses are, in another interesting way, not necessarily mutually exclusive. This oversight has arisen largely through a failure to distinguish clearly between *proximate* and *ultimate* causation in answering questions of the general type: ' why do animals do what they do?' (Wittenberger, 1981). *Proximate* answers deal with immediate, physiological mechanisms underlying a behaviour, while answers at the level of *ultimate* causation address the evolutionary reasons why these mechanisms evolved. Hence, a secondary compound may be involved in the proximate choice of a plant, as a token stimulus (Dethier, 1954) indicating to an insect that it has encountered a plant containing the balance of nutrients to which it is best adapted. In this case secondary compounds determine food choice on the behavioural (proximate) level, but the evolutionary (ultimate) benefit is through the nutrient characteristics of the host plant. Similarly, when an insect obtains some secondary advantage from eating plants containing a toxic compound, it does not necessarily follow that this is the

compound to which the insect responds behaviourally (Dethier, 1972). If, for example, the toxin is beyond the sensory reach of the insect, natural selection may favour a behavioural response to a second substance which predictably co-occurs in plants with the toxin. This second compound then serves as a token stimulus indicating the presence of a useful substance in potential food plants.

This concept of the token stimulus may be taken further by considering other aspects of the host plant to which an insect is adapted. The entirety of the relationship existing between host-specific insects and the plants on which they live has perhaps best been expressed in Kennedy's (1953) laconic statement: ".....the favoured host plant is not merely something fed on, it is something lived on". An insect is thus adapted to a multitude of factors related to the plant it parasitises, including nutrition, specific plant chemicals, phenology, morphology, associated predators, and even to the plant habitat and ecological aspects imposed by other plants in the community (Dethier, 1972; Gilbert and Singer, 1975; Atsatt and O'Dowd, 1976; Wratten and Edwards, 1980; Holdren and Ehrlich, 1982). A secondary compound may therefore signal to the insect not only that it has located an adequate source of food, or a means of biochemical defence, but that it has located the niche to which it is adapted.

CYANOGLYCOSIDES IN PLANTS

The synthesis and storage of compounds capable of releasing cyanide upon hydrolysis (i.e. of cyanogenic compounds) is a widespread phenomenon among plants, known to occur in at least 2050 species representing 110 families (Seigler, 1981). Of the three chemical types associated with this cyanogenic capability, the cyanoglycosides, cyanolipids and pseudocyanogenic glycosides, cyanoglycosides are by far the most widespread. Cyanolipids are known to occur only in the Sapinadaceae, and are thought to occur in one genus of Boraginaceae (Seigler, 1981). Pseudocyanogenic glycosides have thus far been detected only in the Cycadaceae (Seigler, 1975). Their ubiquity, together with the considerable information acquired in recent years on their metabolism in both plants and animals, make the cyanoglycosides a group of secondary plant chemicals imminently suited to the study of plant-herbivore interactions (Conn, 1979).

Despite the widespread occurrence of cyanoglycosides, the structures of only about 55 of these compounds have been reported to date and the compounds responsible for cyanogenesis have been studied in only about 200 plant species (Seigler, 1981). These limited data are partly a result of difficulties in their isolation and purification, and the fact that cyanoglycosides are decomposed by enzymes released upon damage to the plant tissue in which they occur (Seigler, 1975, 1981).

The general features of the biosynthetic pathway of cyanoglycosides in plants have been well established (Butler and Conn, 1964; Conn, 1981). This pathway involves the sequential conversion of an amino acid precursor, via aldoxine and nitrile intermediates, to an ∝-hydroxynitrile (cyanohydrin). In sorghum at least, this biosynthetic sequence is catalyzed by a membrane-bound enzyme system closely associated with the endoplasmic reticulum of the cell (Saunders *et al.*, 1977). The ∝-hydroxynitrile is then glycosylated yielding a cyanoglycoside.

The known cyanoglycosides fit into five groups according to biosynthetic origin (Seigler, 1981). The majority of these, comprising four of the structural groups, have aglycones derived from five proteinaceous amino acids: valine, isoleucine, leucine, phenylalanine, and tyrosine. The fifth group, those having a cyclopentenoid aglycone, are probably derived from the non-protein amino acid cyclopentenyl glycine (Conn, 1981). The distribution of these structural types within the plant kingdom is of potential chemotaxonomic importance; a fact which is particularly true of the cyclopentenyl group, presently known to occur only in the closely related Flacourtiaceae, Passifloraceae and Turneraceae (Saupe, 1981).

Despite the difficulty in demonstrating a protective function for plant secondary substances (Feeny, 1975), convincing evidence now exists that cyanoglycosides are effective herbivore deterrents for some species at least. The first clear demonstration of this was by Jones (1962) who found that some mollusc species, when offered a choice between cyanogenic and acyanogenic strains of *Lotus corniculatus* (L.), preferentially ate the cyanogenic form. This evidence has subsequently been corroborated by several studies both in this field (Angseesing and Angseesing, 1973; Cooper-Driver and Swain, 1976; Lawton, 1976) and in the laboratory (Woodhead and Bernays, 1977). Miller *et al.* (1975) could, however, find no evidence that herbivores feed selectively on acyanogenic white clover seedlings and therefore concluded that herbivory is not the chief selective pressure maintaining cyanogenesis in this species. Jones *et al.* (1978) criticised this conclusion on the grounds that, *inter-alia*, it was not established whether the

herbivores used in the study were specially adapted to cyanogenic plants or not. If this was the case then it could be argued that other, non-adapted, herbivores are deterred and this would provide an adequate selective basis for the maintenance of cyanogenesis in the white clover.

It has long been suspected that the protective function of cyanoglycosides is due to their ability to release HCN upon enzymatic hydrolysis (cyanogenesis) (Jones *et al.*, 1978; Davis, 1981). The enzymology of this process has been extensively studied and is now well understood (Hösel, 1981). On disruption of the plant tissues in which they occur (as happens during attack by an herbivore) spatial separation of β-glycosidase enzymes and the cyanoglycosides is broken down. In sorghum the cyanoglycoside is stored in vacuoles and the enzyme system is probably present in the cytoplasm (Saunders *et al.*, 1977); although there is evidence that in *Trifolium repens* the enzymes are associated with the cell walls (Hughes and Dunn, 1982). The mixing of hydrolytic enzymes and cyanoglycosides results in the hydrolysis of the latter, yielding a sugar and an α-hydroxynitrile. Depending on the pH of the medium, the aglycone may then decompose spontaneously or an α-hydroxynitrile lyase catalyses the reaction which yields a carbonyl component and HCN (Davis and Nahrstedt, 1985). These enzyme systems show various degrees of specificity according to the structural type of cyanoglycoside produced by the plant in which they occur (Hösel, 1981).

Hydrogen cyanide is a potent metabolic poison, the best known effect of which is respiratory inhibition through combining with the terminal oxidase (cytochrome oxidase) of the mitochondrial respiratory chain. There are also several other enzyme systems known to be as, or even more, sensitive to inhibition by HCN as cytochrome oxidase (Solmonson, 1981). The toxic potential to herbivores of this compound is borne out by the numerous reports in the literature of cyanide poisoning resulting from the consumption of cyanogenic plants by livestock (Hurst, 1942; van der Waldt, 1944; Kingsbury, 1964) and by man. Indeed it has been estimated that HCN has probably accounted for more human fatalities than any other chemical known, particularly because of its use as a genocidal agent in World War II (Way, 1981). It has also been demonstrated that HCN is an effective deterrent to insect herbivores (Cooper-Driver and Swain, 1976; Bernays *et al.*, 1977; Woodhead and Bernays, 1977).

Notwithstanding the apparent importance of HCN, other aspects of the toxicity of cyanoglycosides warrant attention in the study of plant chemical defences. Thus Conn (1979) warned that the toxic potential of the carbonyl group resulting from

the hydrolysis of cyanoglycosides - particularly the aromatic aldehydes and their acid oxidation products - should not be overlooked. Also, evidence exists to suggest that the intact cyanoglycoside may be the deterrent to some herbivores (Nayar and Fraenkel, 1963; Braekman *et al.*, 1982). Indeed, some have expressed doubts about the efficacy of HCN as a defence against herbivory (Jones *et al.*, 1978). One credible basis for such reservations is the fact that so many organisms, ranging from bacteria to man, have enzyme systems capable of detoxifying cyanide (Jones *et al.*, 1978; Davis 1981). A major mechanism for such detoxification is through conversion to thiocyanate, catalyzed by the enzyme rhodanese.

It is therefore clear that, although we can now accept a defensive role for cyanoglycosides against some herbivores at least, their action is by no means absolute . Furthermore, the pharmacological basis of their toxicity is not clearly understood, and probably differs for different species and different structural types of these compounds. Nonetheless, considering their widespread occurrence, and that there is a cost involved in producing cyanoglycosides (Jones *et al.*, 1978), it seems reasonable to conclude that herbivory has been a major selective force in maintaining the production of these compounds as defensive chemicals in plants.

CYANOGLYCOSIDES IN LEPIDOPTERA

Compared with the number and broad taxonomic distribution of cyanogenic plant species, production of cyanide by animals appears to be fairly restricted (Duffey, 1981). Cyanogenesis has only been observed in a single animal phylum, the Arthropoda, within only three of the 11 extant classes. In only one of these - the Hexapoda - have cyanoglycosides been detected; in the remaining two classes, the Diplopoda and Chilopoda, cyanide appears to be released from various nitriles (Eisner, 1970; Duffey, 1981).

The majority of known cyanogenic insects have been discovered within the Lepidoptera (Davis and Nahrstedt, 1985), although cyanoglycosides have also been detected in Heteroptera (Braekman *et al.*, 1982) and Hymenoptera (Davis and Nahrstedt, 1985) and are thought to exist in Coleoptera (Moore, 1967) . Within the Lepidoptera, these compounds have thus far been identified in three of the seven subfamilies of Zygaenidae, and from two taxa in the Nymphalidae, the Heliconiini and Acraeinae (Davis and Nahrstedt, 1985). The study of cyanogenic insects is, however, fairly recent and these substances may yet be found in several other groups.

Jones *et al.* (1962) were the first to report on cyanogenesis in Lepidoptera, when they demonstrated that the tissues of all stages in the life cycle of *Zygaena filipendulae* (L.) and *Z. trifolii* (Esper), and imagines of *Procris geryon* (Hueb) (also in the Zygaenidae) release cyanide when crushed. Following this discovery Brower and Brower (1964) predicted, on the basis of natural history and the known chemistry of their food plants, that *Heliconius* butterflies would sequester cyanoglycosides from the *Passiflora* plants on which they feed. This prediction was later extended by Rothschild (1971) to the Acraeinae, which are also closely associated with the Passifloraceae (Ackery, 1987). Owen (1970) had at that stage already stated his observation that the thoracic exudate of *Acraea encedon* does indeed release HCN.

The most revealing report to that point on a compound structurally related to cyanoglycosides in Lepidoptera arose from the study of Teas (1967). This study demonstrated that a species of arctiid moth, *Seirarctia echo* Abbot and Smith, sequesters cycasin, the glycoside of methylhydroxymethanol (a so-called pseudocyanogen - Seigler, 1975) from its *Cycas* host plant. The metabolic process postulated to account for this sequestration is that cycasin from the plant is hydrolysed in the insect gut, and the aglycone diffuses into the moth's tissues. There it is reconverted to cycasin, the storage product, which remains dissolved in the insect's body fluids. Both cycasin and its aglycone are potent toxins, as well as carcinogens and mutagens for vertebrates (Teas, 1967).

It was not until 1979 that cyanoglycosides were first detected and identified in Lepidoptera (Davis and Nahrstedt, 1979). These authors detected linamarin and lotaustralin, the valine and isoleucine-derived cyanoglycosides respectively, in imagines of *Z. filipendulae*. It has subsequently been shown that at least 45 species of Zygaenidae contain these glycosides, and that in at least two species they are present in all stages of the life cycle (Davis and Nahrstedt, 1982, 1985). Interestingly, linamarin and lotaustralin are widespread among the food plants of Zygaenidae, particularly those in the Papilionaceae (Davis and Nahrstedt 1979). However, the observation that some species feeding on non-cyanogenic plants contain linamarin and lotaustralin (Jones, 1962; Davis and Nahrstedt, 1979, 1985), led these authors to suggest that *Zygaena* species are capable of synthesising linamarin and lotaustralin from valine and isoleucine, their respective amino acid precursors.

Following the pioneering work on Zygaenidae, Nahrstedt and Davis (1981) detected linamarin alone or linamarin and lotaustralin in imagines of five species of *Acraea* and three species of *Heliconius*. It has since been demonstrated that both

the imagines and pupae of *Acraea violae* (Fabricius) contain linamarin and that six additional genera of Heliconiini have species containing both glycosides (Nahrstedt and Davis, 1983). Since most of these species feed on Passifloraceae and related families, and these plants are known to produce cyclopentenyl cyanoglycosides, which are biosynthetically unrelated to linamarin and lotaustralin, these authors suggested that *Acraea* and heliconiine butterflies must synthesise *de novo* the cyanoglycosides contained in them. *De novo* synthesis has subsequently been proved for *Heliconius melpomene* (Nahrstedt and Davis, 1983) and *Zygaena filipendulae* (Wray *et al.*, 1983) when it was shown that these insects incorporate radioisotope-labelled valine and isoleucine into linamarin and lotaustralin, respectively.

It is now known that about 70% of the cyanoglycoside in *Z. trifolii* larvae is concentrated in the integument, including the cuticular cavities, and almost all of the remaining 30% is in the haemolymph (Davis and Nahrstedt, 1985; Franzl *et al.* 1986). Despite this high concentration in the integument it has not been possible to show, using electron microscopical techniques, that these insects have any special morphological adaptations in epidermal cells for secretory activity (Franzl and Naumann, 1985). Recent evidence suggests that the site of synthesis of linamarin and lotaustralin in *Z. trifolii* is within the larval tissues, including the fat body, the gut and possibly the haemolymph (Franzl *et al.*, 1986).

Nahrstedt and Davis (1985) comment that although available evidence suggests that *Zygaena* synthesise linamarin and lotaustralin, this does not exclude the possibility that they may obtain these compounds from their foodplants as well. In further investigating this possibility, Nahrstedt and Davis (1986) found a positive correlation between the linamarin : lotaustralin ratio of individual food plants from natural populations and that of *Z. trifolii* and *Z. filipendulae* larvae feeding upon them. It was further shown that this ratio could be altered by feeding *Z. trifolii* larvae acyanogenic leaves impregnated with linamarin or lotaustralin. Final proof that *Z. trifolii* can sequester these compounds was provided when larvae fed (aglycone ^{14}C) glycosides were shown to accumulate these in their tissues. The authors suggested that the metabolic process of sequestration may be similar to that postulated for the sequestration of cycasin by *Seirarctia echo* (Teas, 1967).

Cyanoglycosides are generally assumed to have a defensive role in insects, but the evidence supporting this is currently circumstantial. One line of evidence is based on the fact that both HCN and cyanoglycosides are bitter tasting, to man at least (Bate-Smith, 1972). This is probably also true for a wide range of vertebrate insect predators, since the sense of taste is remarkably uniform throughout the

animal kingdom (Brower, 1984). Vertebrates generally respond to bitterness, even at extremely low concentrations (Moncrieff, 1967), with aversion and this response is probably highly adaptive since virtually all biological toxins are bitter tasting (see Brower, 1984 for refs.). This generalisation has been borne out for several species of insectivorous birds, all of which showed a negative response to food to which bitter compounds had been added (Brower and Glazier, 1975; Eisner *et al.*, 1978). Furthermore, a vertebrate's aversion to cyanoglycosides need not be based on bitterness alone since their ingestion may cause severe stomach pain, vomiting and even death (Bowman and Rand, 1980). These symptoms are due to the blockage of intracellular respiration by HCN which is released on hydrolysis of the glycosides in the acid environment of the stomach. A second line of evidence is that some species of insect known to contain cyanoglycosides - e.g. *Heliconius* and *Acraea* butterflies and *Zygaena* moths - also have warning colouration, indicating the presence of a toxic substance (Marsh and Rothschild, 1974). It has also been shown that *Acraea* butterflies are unpalatable to birds (Swynnerton, 1919) and monkeys (in Carpenter, 1921), and that some species of *Heliconius* are unpalatable to birds (Brower and Brower, 1964). Finally, since cyanoglycosides in plants are known to deter herbivores (Jones *et al.*, 1978), it is tempting to conclude that they will be equally effective against insectivores (e.g. Eisner, 1970 p.201).

It therefore appears that, although a strong case can be made for a protective function of cyanoglycosides in insects, this has yet to be confirmed by direct evidence. In particular, what is needed is direct evidence bearing on the actual consequences for fitness of cyanogenesis in insects. Until this is available we face the danger, forcefully spelt out by Duffey (1981 p.412), that our interpretation of the biological function of cyanogenesis may be "....dictated by teleological explanations of what chemicals should do rather than what organisms do". It is significant that this situation has not changed for the study of cyanogenic insects since Brower and Brower in 1964 raised the topic for plant-derived insect defences in general: "....it must be emphasised that nearly all the evidence has been obtained from anecdotal accounts or from experiments conducted without adequate controls. As a result it has not been possible to make quantitative statements regarding the comparative palatabilty of the numerous species studied. Yet this is a situation greatly to be desired ..... to provide a solid groundwork for biochemical investigation of the unpalatability of insects in relation to the plants which they eat...." (Brower and Brower, 1964 p.138). What is therefore needed for insects, as Jones (1971) pointed out for cyanogenic plants, is a situation where both a cyanogenic and an acyanogenic strain of the same species are available for the design of properly controlled experiments.

OVERVIEW AND REASONS FOR THE PRESENT STUDY

Recent developments in chemical techniques have allowed considerable advances in our understanding of plant secondary compounds, their origins, and their relation to herbivorous animals. It is now clear that these substances have played a role in the evolution of patterns of host utilisation by herbivores, in some groups of plants and insects at least. It has also been established that the utilisation of plant-produced chemicals by herbivorous insects, both in host-recognition and, through their sequestration, as predator deterrents, is a widespread phenomenon. It is, however, uncertain how pervasive the role of secondary compounds has been in the evolution of patterns of host utilisation by phytophagous insects. What is therefore needed are in-depth studies for a wide range of insects, plant taxa and secondary compounds on the role of these substances in mediating the interactions between plants and phytophagous insects.

Cyanoglycosides are an interesting group in this respect. Firstly, they are widespread among plants and such convergence suggests a strong selective advantage in their production. It is also now well established that they have a defensive role against herbivores, although the defensive role of cyanoglycosides in insects has yet to be further investigated . Secondly, their biosynthesis and metabolism in both plants and insects are now well understood. Thirdly, and perhaps most interesting, is the observation that cyanogenic Lepidoptera which feed on cyanogenic plants (e.g. Heliconiini and several Acraeinae and Zygaenidae) synthesise cyanoglycosides themselves. In this they are an important exception since phytophagous insects that contain the same toxins as their hosts usually obtain these from the plants. It has therefore been suggested that cyanogenic Lepidoptera are unique among known toxic insects because their toxicity preceded their association with toxic plants (Rothschild, 1972; Davis and Nahrstedt, 1985). That is, their ability to synthesise cyanoglycosides pre-adapted them to cope with these toxins in plants and thus provided these insects with a vacant niche.

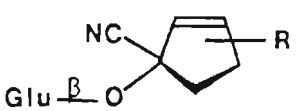
From this it is clear that cyanogenic Lepidoptera are an interesting group in the study of plant-insect interactions and I therefore initiated the present study into biochemical aspects of the association between the aposematic butterfly *Acraea horta* L. (Acraeinae), and its cyanogenic food plant, *Kiggelaria africana* L. (Flacourtiaceae). This work is described in the three chapters which follow, each of which is written as a primary research report in the format of a journal to which their respective topics are relevant. Chapter 1 describes the isolation, purification and characterisation of gynocardin, the cyanogenic compound from the

leaves of *K. africana*. In chapter 2 I describe the discovery that *A. horta* imagines also contain gynocardin and present evidence that, unlike any nymphalid butterfly hitherto studied, these insects sequester this cyanoglycoside from their larval food plant. This has important implications for our interpretation of how acraeine butterflies came to be associated with cyanogenic plants, and chapter 2 includes a discussion of these implications. If *A. horta* obtains a toxic substance from leaves the larvae eat, then the presence of this compound may be an important factor influencing host choice. Chapter 3 describes an investigation into the role of cyanoglycosides in host selection by *A. horta* larvae. Although larvae did indeed select cyanogenic species from amongst potential food plants, the chemical basis of this selectivity was not the cyanoglycoside itself. This suggests that *A. horta* may provide an interesting example of insects which use a token stimulus to recognise the presence of toxic compounds, in plants, from which they derive a secondary advantage.

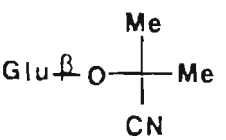
CHAPTER 1

GYNOCARDIN FROM THE LEAVES OF *KIGGELARIA AFRICANA* L. (FLACOURTIACEAE)

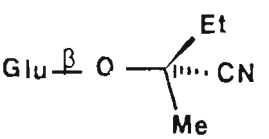
Kiggelaria africana L. is a member of the Flacourtiaceae, and the glucosides of cyanohydrins of substituted cyclopentenones (formula 1) are found exclusively in this and the taxonomically related families, Passifloraceae and Turneraceae (Seigler, 1981). These families are also the major group of foodplants of *Acraea* spp. and *Heliconius* spp. (Lepidoptera: Nymphalidae) (Nahrstedt and Davis, 1981), but several species of both genera of butterflies contain the acyclic cyanoglucosides linamarin (formula 2) and lotaustralin (formula 3) (Nahrstedt and Davis, 1981, 1983).



1



2



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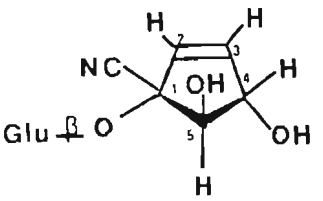
Since linamarin and lotaustralin are unrelated to the cyanoglucosides in the larval food plants of *Heliconius* and *Acraea* butterflies, it has been suggested that these glucosides are synthesised *de novo* in the insects. This has moreover been proved for *Heliconius melpomene* (L.) when Nahrstedt and Davis (1983) demonstrated that larvae and imagines incorporate radioisotope-labelled valine and isoleucine into linamarin and lotaustralin, respectively. In contrast, it has recently been demonstrated (Chapter 2) that *Acraea horta* (L.) contains a cyanoglucoside different from linamarin and lotaustralin and identical with that of their food plant, *K. africana*. This was the first indication of a plant-produced cyanoglucoside in *Acraea* butterflies and it was therefore of interest in the present study to isolate and identify the cyanoglucoside in *K. africana*.

Fresh leaves of *K. africana* were extracted using a modification of the conventional procedure. Repeated chromatography of the cyanogenic fractions, as

monitored by an enzymic procedure (Tantisewie *et al.*, 1969; Spencer and Seigler, 1982), finally yielded gynocardin (formula 4). Characterisation was by means of spectroscopic data which agree with the values reported in the literature (Coburn and Long, 1966; Hübel *et al.*, 1981).



4

EXPERIMENTAL

General experimental procedures

Melting points were measured on a Fisher-Johns apparatus. All solvents were redistilled before use. Pet. ether refers to petroleum ether, boiling range 60-85 °C. Silica gel (Merck 70-230 mesh) was the stationary phase used in chromatographic columns, and 35-70 mesh was used as an adsorbate for syrups. Glycosides were monitored on thin layer chromatograms (tlc) (silical gel 60F 254) by spraying with cerium(IV) sulphate in 1.5M H_2SO_4 followed by heating for 1-2 minutes on a hot plate. Glycoside-positive fractions were then monitored for cyanogenesis by spraying either tlc plates or paper chromatograms with a crude enzyme extract (Spencer and Seigler, 1982) of leaves of *K. africana* and overlaying the surface first with medicinal gauze then with a sheet of filter paper previously sprayed with a solution of picric acid and sodium carbonate (Tantisewie *et al.*, 1969). The whole was sandwiched between two glass plates, sealed with masking tape, and incubated overnight at approx. 30 °C. The cyanoglucosides gave red spots on the picrate paper. Tlc solvent mixtures were the following:

A. EtOAc-Me_2CO-MeOH (4:4:1); B. $CHCl_3$-MeOH (5:2); C. CH_2Cl_2-EtOH (2:1).
For paper chromatograms (Whatman No. 1, descending), the following solvents were

used: D. MeCOEt-Me_2CO-H_2O (15:5:3); E. Me_2CO-H_2O (5:1); F. n-BuOH-AcOH-H_2O (2:1). Infra-red (i.r.) spectra were recorded on a Perkin-Elmer 983 i.r. spectrophotometer; ^1H nmr and ^{13}C nmr spectra were recorded on a Varian VXR 200 spectrometer; mass spectra were recorded on a VG Micromass 16F coupled with a VG Data System 2000; optical rotations were measured on a Perkin Elmer 141 spectropolarimeter. The term "rotavap" refers to evaporation on a rotary vacuum evaporator.

Plant material

Leaves of *K. africana* were obtained locally, and a voucher specimen has been deposited with the Bolus Herbarium, University of Cape Town.

Extraction and isolation

Fresh leaves (500g) were macerated in a Waring blender with 96% ethanol and the slurry was boiled rapidly for 3-5 minutes, cooled and filtered. The solids were washed with ethanol and the clear filtrate was rotavapped to a syrup which was dissolved in an equal volume of water and partitioned successively with equal volumes of pet. ether then dichloromethane. The defatted aqueous extract was again rotavapped to a syrup (29g). A portion (10g) was adsorbed onto silica gel (10g), powdered and placed on top of a column of dry silica gel (500g). Me_2CO-H_20 (5:1) was used to elute the column and 15ml fractions were collected. These were monitored and the contiguous cyanoglucoside-containing fractions were combined, rotavapped to a syrup, and rechromatographed on a second silica gel column using $CHCl_3$-MeOH (5:2). Evaporation of the cyanogenic fractions gave gynocardin (formula 4), melting point (m.p.) 157-160 °C (from i-PrOH) $[\alpha]_D$ + 84.2 °(c 0.25 MeOH) [literature, Coburn and Long (1966): m.p. 165-166 °C, $[\alpha]_D$ +72.9 °(water)]. ^1H nmr, $\delta(D_2O$, 30 °and 80 °C) 6.27 [dd, J_{23} 8Hz, J_{34} 2Hz, -C(2)H=C(3)H-CH], 6.04 [d, J_{23} 8Hz, - C(2)H=CH-], 4.81 (d, J J 8Hz, Glu (β)-C(1)H], 4.67 [dd, J_{45} 5.5Hz, -C(4)H-], 4.32 [d, J_{45} 5.5Hz,-C(5)H-] and 3.3-4.0 ppm [6H, m, Glu C2'-C6']; ^{13}C nmr, δ(dioxane) 139.9 and 127.4 [-C(2)=C(3)-], 115.6 (-CN), 99.2 [Glu(β)-C(1')], 86.5 [-C(5)OH-], 84.7 [NC-C(1)-], 77.7 [-C(4)OH-], 76.1, 75.5, 72.8, 69.7 [Glu C(2')-C(5')] and 60.8 ppm [Glu C(6')] [assignments were confirmed in the APT spectrum and agree satisfactorily with literature values (Hübel *et al.*, 1981)] [Found: C 47.1, H 5.95, N 4.6%. Calculated for C_{12} H_{17} NO_8: C 47.5, H 5.6, N 4.6%].

Gynocardin hexaacetate

Gynocardin (30mg) was treated with dry pyridine (1.0ml) and acetic anhydride (1.0ml) and left overnight. The mixture was poured onto ice (10g) and extracted with dichloromethane (5 x 5ml). The combined organic extracts were successively washed with saturated $NaHCO_3$ solution (10ml) and water (10ml) then dried (Na_2SO_4). The solvent was removed and high vacuum applied to remove traces of pyridine to leave crude gynocardin hexaacetate, m.p. 117-119 °(from pet. ether-ethyl acetate) $[\alpha]_D$ + 36.7 ° (literature, Coburn and Long (1966): m.p. 120 °C; $[\alpha]_D$ +40.4 °($CHCl_3$)], ^1H nmr, ($CDCl_3$) 6.20 [dd, J_{23} 5.9 Hz, J_{34} 1.9Hz, -C(2)H=C(3)H-C(4)H], 5.98 [d, J_{23} 5.9Hz, -C(2)H=C(3)H-], 5.65 [d, J 2.5Hz, -C(5)H-], 5.54 [m, -C(4)H-], 5.17 ([, J 9.0Hz, Glu -C(1')H], 4.88 - 5.05 [3H, m, Glu C(2')-C(4')], 4.11 [2H, d, J 3.7Hz, Glu -CH_2OH] and 3.75 ppm [m, C(5')]; ms, m/z (% rel. int.) 331 (7), 259 (12), 250 (42), 208 (18),199 (10), 169 (44), 157 (26), 150 (29), 139 (31), 115 (26), 109 (33), 97 (26), and 43 (100).

Acidic hydrolysis of gynocardin

Gynocardin (2mg) was heated with 2M trifluoroacetic acid (TFA, 1ml) in a sealed tube for 6 hrs at 100 °C. After cooling, the contents of the tube were rotavapped 5 times with methanol to remove the TFA. The residue was applied to a sheet of Whatman No. 1 paper with *D*-glucose as standard and chromatographed in solvent system F overnight. The dried paper after spraying with p-anisidine hydrochloride in n-BuOH and heating, showed a single spot identical with *D*-glucose.

DISCUSSION

The data for the unknown agreed well with those previously reported for gynocardin (Coburn and Long, 1966; Hübel *et al.*, 1981). Gynocardin was first isolated from the seeds of *Gynocardia odorata* (Flacourtiaceae) (Power and Gornall, 1904), and the known distribution of this compound has now been extended to *K. africana*. Since both plants are members of the Flacourtiaceae, this provides additional evidence for the value of cyclopentenoid cyanogens as phylogenetic markers (Saupe, 1981).

CHAPTER 2

THE CYANOGLYCOSIDE GYNOCARDIN FROM *ACRAEA HORTA* (L.) (LEPIDOPTERA: ACRAEINAE): IMPLICATIONS FOR THE EVOLUTION OF ACRAEINE HOST CHOICE

ABSTRACT.1. All stages of the life cycle of *Acraea horta* (L.) (Lepidoptera: Acraeinae) were found to release hydrogen cyanide (HCN) from their crushed tissues, and the cyanogenic substance occurred in the haemolymph of imagines.

2. Comparison with standards on thin layer chromatograms (tlc) revealed that the chemical source of HCN in imagines is the cyanoglycoside gynocardin, which is also produced by the larval food plant, *Kiggelaria africana* L. (Flacourtiaceae).

3. Analysis of imagines reared on plant species (Passifloraceae) containing gynocardin and/or other cyanoglycosides suggested selective uptake of gynocardin by *A. horta.*

4. This is the first demonstration of a cyanoglycoside other than the acyclic linamarin and lotaustralin occurring in Lepidoptera, and the first suggestion of toxic substances being sequestered by nymphalid butterflies.

5. This evidence indicates that the association of *Acraea* butterflies with cyanogenic plants preceded the cyanogenic capability of these insects.

INTRODUCTION

Unpalatable insects often obtain toxins from the plants they eat (Brower and Brower, 1964; Rothschild, 1972). It was therefore surprising when evidence emerged suggesting that all three groups of Lepidoptera known to contain cyanoglycosides (Zygaenidae and the nymphalid groups, Heliconiini and Acraeinae) are an exception in this regard. The first evidence was reported by Jones *et al.* (1962) who found that *Zygaena fillipendulae* (L.) and *Z. lonicerae* (von Schev.) (Zygaenidae) release HCN from their crushed tissues, even when reared on acyanogenic plants. Nahrstedt and Davis (1979) subsequently detected linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile) and lotaustralin (2-β-D- glycospyranosyloxy-2-methyl- 2R-butyronitrile) - the same two glycosides produced by their larval food plants - in imagines of *Zygaena*

filipendulae. Thereafter one or both of these compounds were detected in the imagines of five species of *Acraea* and three species of *Heliconius* (Nahrstedt and Davis, 1981); and subsequently in the imagines and pupae of *A. violae* (Fabricius) and in six additional genera of Heliconiini (Nahrstedt and Davis, 1983). Since the food plants of Heliconiini (Passifloraceae and Turneraceae) (Smiley, 1985) and the major food plants of *Acraea* (Passifloraceae, Flacourtiaceae and Turneraceae) (Ackery, 1987) produce cyanoglycosides with a cyclopentenyl moiety, which are structurally unrelated to those in the insects, it was concluded that linamarin and lotaustralin are synthesised by these nymphalid butterflies. Nahrstedt and Davis (1983) confirmed this for *Heliconius melpomene* (L.) by demonstrating that larvae and imagines incorporate radioisotope-labelled amino acids into linamarin and lotaustralin.

The question thus arises, why do cyanogenic Lepidoptera feed on cyanogenic plant species if not to obtain defensive chemicals? The recent discovery that *Zygaena trifolii* (Esper) and probably *Z. filipendulae* are indeed capable of sequestering linamarin and lotaustralin from their food plants, in addition to synthesising these compounds (Nahrstedt and Davis, 1986), provided a plausible explanation for Zygaenidae. Of the Heliconiini and Acraeinae, it has been suggested that these groups were able to colonise the largely competitor-free cyanogenic plants because their already established ability to synthesise cyanoglycosides demanded that their tissues be immune to the toxicity of these compounds (Rothschild, 1972; Davis and Nahrstedt, 1985). However, this hypothesis fails to explain why several species of *Acraea* show the typical patterns of host utilisation characteristic of insects which obtain defensive chemicals from the plants they eat (Rothschild, 1972). These patterns include different subfamilies of insects being more or less confined to one group of plants, e.g. Heliconiini and Acraeinae feeding on Passifloraceae and related families; and groups of insects (e.g. Acraeinae and to a lesser extent, Heliconiini) which feed on several families of plants with taxonomic and/or chemical affinities.

Acraea horta (L.) is a southern African species which is monophagous on *Kiggelaria africana* L. (Flacourtiaceae) in its natural habitat, but is also known to feed on introduced garden plants of the Passifloraceae (Pennington, 1978). In chapter 3 it is reported that this species was found to feed on several additional Flacourtiaceae and Passifloraceae - but only on the cyanoglycoside-producing members of these families. The Passifloraceae are also the major food plants of Heliconiini; and the Flacourtiaceae

and Passifloraceae are taxonomically related (Heywood, 1978). They also have biochemical affinities in that they both produce cyanoglycosides with a cyclopentenyl moiety (Seigler, 1975). The feeding patterns of *A. horta* thus resemble those characteristic of insects which obtain toxins from their food plants, and the purpose of the present study was therefore to investigate the source of cyanoglycosides in this species.

METHODS AND MATERIALS

Eggs and pupae were tested for cyanogenesis by crushing the intact organisms in approx. 3ml of distilled water in a glass vial (50 x 10mm) in which a strip of picrate paper was suspended (Steyn, 1934). The same test was used for larvae, but only after the digestive tract had been dissected out to remove any residual plant tissue. The dissected wings, head and thorax, and abdomen of the imagines were tested separately. In addition, haemolymph was carefully removed from the wing veins of adults using a glass micropipette (2 micro litre) and tested for cyanogenesis in the same way. The presence of HCN is indicated by a change in the picrate paper from yellow to purple-brown, and the time taken for this change to begin and the final intensity of the red colouration are roughly proportional to the quantity of HCN released (Jones *et al.*, 1962; Gibbs, 1974). In all cases the test was allowed to run for 24 hours.

Extraction of plant and animal tissues was performed as previously described (chapter 1). Correlative chromatography was done using methanolic solutions of the cyanogenic substances on tlc plates (silica gel 60F, 254), and Whatman No. 1 paper. Reference standards were pure gynocardin as previously described (chapter 1) and a sample of linamarin (from Professor A. Nahrstedt who reported that it contained approx. 1% lotaustralin; this, however, was not detectable by tlc). Cyanoglycosides were monitored using both cerium(IV) sulphate reagent for glycosides and by hydrolysis with crude enzyme extracts and detection using an overlay of picrate paper (chapter 1).

Eggs, larvae, pupae and imagines were collected from *K. africana* trees in Newlands Forest, Cape Town (33°57'S; 18°27'E). Leaves of *Passiflora caerulea* L., *Tacsonia mollissima* Kunth. and *Tacsonia manicata* Juss. were obtained from gardens in Cape Town. Identification of these plants were confirmed by Mr C.G.C. Dickson, and voucher specimens have been deposited in the Bolus Herbarium, Cape Town.

Larvae were reared in plastic dishes (645 x 75 x 110mm) in a growth chamber maintained at 20 °C and 65% relative humidity with a 12/12 light/dark cycle. Fresh leaves were provided daily.

Identification of gynocardin in A. horta

235 (16g) frozen *A. horta* imagines, collected between 12.3.1986 and 2.2.1987, were extracted with 96% ethanol and the cyanogenic fractions were isolated using a silica gel column as described in chapter 1 for *K. africana.* These were partially purified using preparative plate chromatography (silica gel 60, Merck; ethyl acetate-acetone-methanol 4 : 4 : 1). The cyanogenic front (R_F 0.38 - 0.62) was located by spraying a 1cm wide strip of the chromatogram with crude enzyme extract from *K. africana* (chapter 1), and overlaying this with medicinal gauze and picrate paper. This was enclosed between glass sheets and incubated at 30 °C until a red band appeared on the picrate paper. This indicated the cyanoglycoside band which was eluted using methanol, and compared with linamarin and gynocardin in several solvent systems using tlc plates and paper chromatography. Details of solvent systems used can be seen in Table 1.

Rearing experiments

To investigate the possible sequestration of cyanoglycosides from the leaves consumed, eggs and second and third instar larvae of *A. horta* collected from *K. africana* trees were reared to imagines on *P. caerulea*, *T. mollissima* and *T. manicata.* These plants were selected as those most readily eaten by fifth instar larvae in a previous laboratory feeding experiment (chapter 3). Three imagines from each batch were extracted together and the extracts of each batch compared for cyanoglycosides with extracts of their respective food plants. Cyanoglycosides of both plants and butterflies were visualised using crude enzyme extracts from the plant in question, as well as from *K. africana.* This ensured that a negative result was due to the absence of cyanoglycoside, and not of appropriate hydrolytic enzymes.

RESULTS

Acraea horta *collected from* Kiggelaria africana

Eggs, larvae, pupae and imagines collected from *K. africana* trees gave a strong positive reaction with picrate paper. In all cases a colour change began within five minutes resulting in a dark purple-brown within thirty minutes. The wings, head and thorax, and abdomen of imagines were all cyanogenic, and so was the haemolymph extracted from wing veins. The cyanoglycosides extracted from imagines migrated more slowly than linamarin on tlc plates, but concurrently with gynocardin for several solvent systems on tlc plates and on descending paper chromatograms (Table 1).

TABLE 1. Mobility of cyanoglycoside extracted from *Acraea horta* imagines compared with linamarin and gynocardin standards.

Chromatographic system	Mobility (R_F)		
	Linamarin standard	Gynocardin standard	Extracted from *A. horta*
TLC			
ethyl acetate-acetone-methanol (4:4:1)	0.49	0.41	0.41
chloroform-methanol (5:2)	0.52	0.34	0.34
butanol-acetic acid-water (2:1:1)	0.63	0.58	0.58
butanol-ethanol-water (4:1:5)	0.52	0.50	0.50
dichloromethane-ethanol (2:1)	0.62	0.53	0.53
PAPER (DESCENDING)			
butanone-acetone-water (15:5:3)	not measured	0.71	0.71
butanol-acetic acid-water (2:1:1)	not measured	0.29	0.29
acetone-water (5:1)	not measured	0.88	0.88

Acraea horta *reared on other species*

Tacsonia manicata: Both the leaves of this species and the imagines of larvae reared on these leaves contained gynocardin as a sole cyanogenic component.

Passiflora caerulea: Leaves of this species contained gynocardin as a minor component (as gauged by intensity of development on tlc plates) and a second, less polar cyanoglycoside as a major component (R_F 0.43, tlc in butanol-acetic acid-water, 2:1:1). Imagines reared from eggs on this species contained only gynocardin. No cyanogenic activity resulted when the enzyme extract from the leaves of this species was added to the frass of larvae, suggesting that the major glycoside was not excreted. This cyanoglycoside, or the glycoside extracted from *T. mollissima* (see below), were not hydrolyzed by the enzyme extract of *K. africana.*

Tacsonia mollissima: No gynocardin was detected from this species. A less polar cyanoglycoside, which ran concurrently with the major cyanogen in *P. caerulea* on tlc plates using butanol-acetic acid-water (2 : 1 : 1), was present. No cyanogenic substance was detected on tlc plates from imagines reared from the egg stage on this species. When four intact imagines were crushed in separate vials containing picrate paper, minor amounts of HCN were released, as visually judged according to both the time taken for a colour change to begin and the final intensity of colour relative to controls reared on *K. africana, T. manicata* and *P. caerulea.* The controls caused a colour change within 5 minutes, producing a dark purple-brown within 30 minutes. No colour change had been observed from experimental animals within 30 minutes, and after 24 hours two had changed to light orange and two were unchanged. Imagines of larvae transferred from *K. africana* to *T. mollissima* in their second instar resulted in a final colour change after 24 hours to dark orange, indicating levels of HCN intermediate to those discussed above. Addition of the enzyme extract from *T. mollissima* caused no further colour change, thus confirming that the results were due to small quantities of cyanoglycosides rather than an absence of appropriate hydrolytic enzymes. It was not possible to quantify these results further since survival rates of larvae on all three species were characteristically low, and hence sufficient imagines were not available.

DISCUSSION

Selective sequestering of cyanoglycosides

Results of this study demonstrate that all stages of the life cycle of *Acraea horta* are cyanogenic. The chemical basis of this in the imagines is gynocardin, which is biogenetically unrelated to linamarin and lotaustralin (Conn, 1979), the cyanoglycosides previously detected in several *Acraea* species (Nahrstedt and Davis, 1981). It cannot, however, be ruled out that trace amounts of linamarin and lotaustralin were also present, and more sensitive techniques (e.g. GLC) are needed to determine this. Gynocardin is also the cyanoglycoside produced by *Kiggelaria africana* (chapter 1), the food plant of *A. horta*, and feeding experiments described here suggest that cyanogenesis in *A. horta* is influenced by the diet of these insects. Thus, imagines reared on *Tacsonia mollissima*, which did not contain gynocardin, showed low levels of cyanogenic activity. It is unlikely that this was a result of some general dietary inadequacy due to *T. mollissima* being alien to the evolutionary history of *A. horta*, since imagines reared on *Passiflora caerulea* and *T. manicata*, both of which contained gynocardin, were strongly cyanogenic. Those that fed as larvae on *K. africana* prior to being transferred to *T. mollissima* showed higher cyanogenic activity than those transferred as eggs, and this could be expected since the former had been exposed to a dietary source of gynocardin. Together this evidence suggests that the source of cyanoglycoside in *A. horta* is the plants on which this species feeds.

T. mollissima is strongly cyanogenic but the glycoside responsible for this did not appear in imagines reared on this species, suggesting selective uptake of cyanoglycosides by *A. horta*. Similarly, of the two cyanoglycosides produced by *P. caerulea*, imagines reared on this species contained only gynocardin. Interestingly, crude enzyme extract of *K. africana* did not hydrolyze the second of these glycosides or the sole cyanoglycoside in *T. mollissima*. Therefore, assuming that the hydrolysis of gynocardin in *A. horta* is catalysed by a similar enzyme system, absorption of these compounds would not contribute to the cyanogenic capability of *A. horta*. Neither of these cyanoglycosides appeared in the frass of larvae fed the leaves of *P. caerulea* and *T. mollissima*, and it must therefore be concluded that they were metabolised by the insects.

There are interesting similarities in these results and those of von Euw *et al.* (1967) who studied *Poekilocerus bufonius* (Klug), an aposematic grasshopper that probably feeds exclusively on Asclepiadaceae in nature. The tissues of *P. bufonius* were found to contain two of the six cardenolides produced by its asclepiad food plant, suggesting that the remaining four were either excreted, metabolised or converted *in vivo*

by these insects. Hoppers reared on non-poisonous plants contained approximately ten times less cardenolide than those reared on toxic plants, and these levels were further reduced by a factor of about seven in the offspring of these insects. That these offspring contained the toxins at all suggests that cardenolides are carried over from the adult to the egg stages, and from the eggs to the larvae. However, von Euw *et al.* (1967) contend that cardenolide levels in both generations are unexpectedly high for storage in the egg alone, and postulate that additional factors such as *de novo* synthesis or bio-accumulation through cannabalism must contribute to these. Similarly, the residual cyanogenic activity in *A. horta* imagines reared on *T. mollissima* in the present study may be due to any one or a combination of these factors, but answers to such questions must await detailed quantitative analysis.

Evolution of host choice

The demonstration of gynocardin in *A. horta* necessitates a reappraisal of the hypothesis (Rothschild, 1972; Davis and Nahrstedt, 1985) that cyanogenic Lepidoptera feed on cyanogenic plants because their ability to synthesise cyanoglycosides pre-adapted them to cope with the toxic effects of these compounds. I suggest that the Acraeinae originally sequestered cyanoglycosides from their food plants and subsequently evolved the ability to synthesise these compounds. Parallel, if circumstantial evidence to support this notion comes from a study of acridoid (Orthoptera) feeding behaviour (Bernays and Chapman, 1978). Two families in the Acridoidea, the Pyrgomorphidae and Romaleidae, are known to sequester toxins from the plants they eat, and both these families also possess primitive morphological features. This led Bernays and Chapman (1978) to speculate that sequestration may be a first step by phytophagous insects in adapting to plant toxins.

A second, and more direct line of evidence comes from an analysis of the feeding patterns of Acraeinae, which shows the choice of host plants by *A. horta* to be primitive among the Acraeinae. Of 23 southern African *Acraea* species for which records of foodplants are available (Platt, 1921; van Son, 1963; Pennington, 1978; R. Prŷs-Jones, personal communication), 63% feed on Passifloraceae and the adjacent Flacourtiaceae and Turneraceae (Violales) and 25% feed on some combination of these families. A single species (*A. anacreon* Trimen) is recorded as feeding on one of these families (Passifloraceae) and additionally on unrelated families (Rosaceae and Fabaceae) and 39% feed on a diverse assemblage of nine families in eight orders and five super-

orders (classification following Heywood, 1978). Although this analysis is based on feeding records of only 23 species, a similar pattern, in which a majority of species feed on Violales, probably exists throughout the Acreainae (e.g. see Ackery, 1987). It is unlikely that this pattern originated through convergence from a diverse group of unrelated host plant families, and so it seems that the Violales are the ancestral hosts of Acraeinae. This is the same conclusion reached by Pierre (1984) in his detailed systematic analysis of this nymphalid subfamily. *A. horta*, the only species of Acraeinae for which evidence of sequestration exists, feeds exclusively on these ancestral host plants, and furthermore, contains a cyanoglycoside of the cyclopentenyl group unique to these families (Seigler, 1981). *A. encedon* L., on the other hand, is believed to synthesise cyanoglycosides since imagines of this species contain linamarin and lotaustralin which do not occur in their larval food food plants (Nahrstedt and Davis, 1981). These insects are also, as far as is known, unique among Acraeinae, and indeed unusual among Lepidoptera, in that they feed on a monocotyledonous family, Commelinaceae (Pennington, 1978). Since, in the words of Ehrlich and Raven (1965), "All utilisation of foods other than dicotyledons by butterfly larvae ..... is assumed to be the result of changes from an earlier pattern of feeding on dicotyledons", this singular choice of host plant by *A. encedon* must be considered derived. Furthermore, there is evidence that *A. encedon* does not store cyanoglycosides generally through the body tissues as does *A. horta*, but stores these substances in specialised thoracic glands. In 1969 Owen and Chanter reported that no cyanogenic activity was detected from the tissues of *A. encedon* imagines, but suggested that an analysis of the yellow foam produced from a gland in the thorax of adult butterflies may yield different results. Owen (1970) subsequently reported that HCN is indeed released from this exudate. Such localisation of toxic substances in insects is considered to be specialised (Brower and Brower, 1964) relative to the state in *A. horta* which contain cyanoglycoside throughout their bodies. This provides an additional link between derived characteristics and *de novo* synthesis of cyanoglycosides in the Acraeinae.

A. horta and *A. encedon* represent two extremes in the evolutionary sequence postulated for the Acraeinae: those that feed exclusively on Passifloraceae and related families and contain cyanoglycosides of the structural group characteristic of these families, and those that feed on monocotyledons and synthesise linamarin and lotaustralin, respectively. *A. oncaea* Hoppfer, *A. calderena* Hewiston and *A. natalica*

Boisduval - all of which contain linamarin and lotaustralin (Nahrstedt and Davis, 1981), and yet are known to feed only on Passifloraceae and related families (Pennington, 1978) - may represent an intermediate stage in this sequence. These butterflies have possibly evolved the biochemical machinery for synthesising linamarin and lotaustralin, without having evolved the sensory or ecological changes necessary for a host change to unrelated plants. If there is an evolutionary advantage in broadening of host range (see Feeny, 1975), there is good reason to believe that the behavioural prerequisites of such a switch are controlled by evolutionarally conservative genes. Wiklund (1975), for example, has shown that choice of host plant by adult and larval *Papilio machaon* are controlled by separate genes. Therefore, a successful host switch would rely on the unlikely event of two simultaneous and independent mutations. Either of these mutations occurring singly would be rapidly selected out of the population, since they would result in eggs being deposited on plants on which the larvae will not feed. There nevertheless exists one possible example of a species that has broadened its host range in this manner, since *A. anacreon* feed on Passifloraceae as well as the unrelated families, Rosaceae and Fabaceae (Pennington, 1978).

Two important questions remain: (1) what was the evolutionary impetus for a switch from sequestration to *de novo* synthesis of cyanoglycosides by acraeine butterflies, and (2) why did this switch involve a change from sequestering cyanoglycosides with a cyclopentenyl moiety to synthesising the structurally unrelated glycosides linamarin and lotaustralin. Of the first question, Bernays and Chapman (1978) have shown that acridoid grasshoppers which sequester toxins from their food plants have significantly longer developmental times than others, and this they consider to be a result of the considerable metabolic expenditure involved in the uptake of toxic chemicals. However, little is known about the cost of *de novo* synthesis of cyanoglycosides, and a full answer to this question must therefore await further analyses. Regarding the second question, it may be significant that linamarin and lotaustralin are also the cyanoglycosides synthesised by the Heliconiini (Nahrstedt and Davis, 1981), and by the taxonomically distant Zygaenidae (Davis and Nahrstedt, 1979, 1982). These are also the most widespread cyanoglycosides among plants (Conn, 1979). Such convergence may be the result of some factor that lowers the cost or increases the benefit of manufacturing these relative to other cyanoglycosides. For example, valine and isoleucine, the respective precursors of linamarin and lotaustralin (Seigler, 1975), may be more freely available in insects and plants than cyclopentenyl glycine, the non-protein amino acid precursor of the cyclopentenyl cyanoglycosides (Conn, 1981).

Relationships among acraeine butterflies are currently uncertain (Ackery, 1987), and the present study has shown the chemistry of these insects to be of potential taxonomic importance. We know the chemical basis of cyanogenesis for only seven of the some 160 *Acraea* species and further studies of the relationship between cyanogenesis and host choice in the Acraeinae are therefore needed.

CHAPTER 3

CYANOGLYCOSIDES AND THE FEEDING RESPONSE OF LARVAL *ACRAEA HORTA* (L.) (LEPIDOPTERA: ACRAEINAE)

ABSTRACT.1. The role of cyanoglycosides in the food choice of *Acraea horta* (L.) (Lepidoptera: Acraeinae) was investigated by testing the feeding response of fifth instar larvae to cyanogenic and acyanogenic plants; and to cyanogenic and acyanogenic fractions chromatographically separated from their natural food plant, *Kiggelaria africana* L. (Flacourtiaceae).

2. Only leaves containing cyanoglycoside were eaten by the larvae.

3. However, larvae preferentially ate the acyanogenic fractions of *K. africana* leaves, demonstrating that *A. horta* responds to a feeding stimulant other than cyanoglycosides.

4. The possibility that *A. horta* larvae recognise the presence of cyanoglycosides in leaves by means of a token stimulus is discussed.

INTRODUCTION

Toxic Lepidoptera often feed on taxonomically and biochemically related groups of food plants (Brower and Brower, 1964). To explain the origins of these patterns of host utilisation, Ehrlich and Raven (1965) proposed the theory of coevolution between plants and herbivores. This theory states that as plants evolved chemical defences (toxic secondary compounds) certain herbivores evolved means of coping with these toxins. Such adapted specialists accrued specific benefits from feeding on toxic plants, including reduced competition from unadapted species and reduced predation through the uptake and storage of noxious compounds. It is therefore important that these herbivores recognise plants containing the specific chemicals to which they are adapted, and there now exist numerous cases where phytophagous insects are known to

recognise their hosts by means of secondary plant compounds (e.g. Schoonhoven, 1972a; Harborne, 1982). There are also several examples of Lepidoptera known to sequester toxins from the plants they eat (e.g. Rothschild, 1972), but there are few known cases in which the defensive chemicals sequestered by Lepidoptera from plants are also used as cues in host recognition. One known example of this is *Pieris brassicae* L., which sequester and store toxic glucosinolates from their cruciferous host plants (Rothschild, 1972) and also use these substances as larval feeding stimulants (Verschaffelt, 1910).

The Acraeinae are a tropical group of aposematic butterflies known to contain cyanoglycosides (Davis and Nahrstedt, 1985). Their major larval food plants are the Passifloraceae and the related Flacourtiaceae and Turneraceae (Chapter 2), several of which are also known producers of cyanoglycosides (Gibbs, 1965). Furthermore, recent evidence (Chapter 2) suggests that at least one species, *Acraea horta* (L.), which is monophagous on *Kiggelaria Africana* L. (Flacourtiaceae) in its natural habitat (Pennington, 1978), obtain these compounds from their food plants. Cyanoglycosides have also been demonstrated to serve as feeding stimulants to insects (Nayar and Fraenkel, 1963; Brattsten *et al.*, 1983). Together these facts led me to postulate a similar situation for *A. horta vis á vis* cyanoglycosides as has been established for *P. brassicae* and glucosinolates. The purpose of the present study was therefore to investigate the role of cyanoglycosides in the food choice of *A. horta*.

METHODS AND MATERIALS

Leaf palatability experiment

To determine the palatability of 19 species of Passifloraceae and Flacourtiaceae to fifth instar *A. horta* larvae, freshly picked leaves of each species were placed in five plastic containers (approx. 1.5 to 2.5g per container) with three larvae in each. The plastic containers (645 x 75 x 110mm) had tightly fitting lids, and were thus air-tight when closed. Fresh leaves were weighed before the experiment, and re-weighed to determine the amount eaten, and replaced at intervals of 22 - 26 hours for six successive days. To reduce evaporative water loss from the leaves, a small square of folded tissue paper attached to the wall of each container was kept moist for the duration of the experiment. In addition a control, from which the caterpillars had been omitted, was set up in a sixth container for each leaf species to determine

weight loss of leaves due to evaporative water loss alone. The experiment was conducted in a growth chamber maintained at 25 °C and 65% relative humidity with a 12/12 light/dark cycle.

Leaves chosen for inclusion in this experiment represented all the species of Flacourtiaceae and Passifloraceae available in and around Cape Town. All Flacourtiaceae and *Adenia* spp. were collected and indentified at Kirstenbosch Botanic Gardens, Cape Town. All *Tacsonia* spp. and *Passiflora* spp. were collected from private gardens, and identification was confirmed by Mr G. E. C. Dickson. Voucher specimens have been deposited in the Bolus Herbarium, Cape Town. The leaves of each species were tested for cyanogenesis using the picrate paper test (Steyn, 1934). To determine whether a negative result was due to the absence in the leaf of hydrolytic enzymes or to the absence of cyanoglycoside, all acyanogenic species were re-tested by adding enzyme extract from *Kiggelaria africana* (chapter 1). Larvae were collected from *K. africana* trees in Newlands Forest, Cape Town. In order to ensure that no diapause larvae (Balinsky, 1974) were used, all larvae were starved for 9 hours and then offered *K. africana* leaves. Only those that fed on these leaves were selected for inclusion in the experiment.

It was not possible to conduct the feeding trials on all species simultaneously, and the experiment was therefore divided into four stages, which were conducted between February and May of 1986 and 1987 (Table 1 shows the species included in each stage). Results were calculated as the weight of leaf eaten (g) (= change of weight of leaf - change of weight due to water loss) per gram of caterpillar per 24 hours. This value was averaged for each experimental replicate over the six days in which the experiment was conducted, and the mean of all five experimental repeats was calculated. In order to control for possible differences in motivational state arising from different climatic histories of caterpillars (Balinsky, 1974) used in each stage, final results (Table 1) were expressed as a percentage of the weight of *K. africana* eaten by caterpillars from each respective batch. Statistical tests were performed as in Siegel (1956) (Fisher Exact Probability Test) and Underhill (1981) (Student-t test).

Artificial feeding experiment

The following experiment was performed to test the hypothesis that gynocardin, the cyanoglycoside known to occur in the leaves of *K. africana* (chapter 1), is an essential feeding stimulant to *A. horta* larvae. The experiment was conducted in three phases as described below:

Phase 1: Leaf extracts of *K. africana* (chapter 1) were chromatographed on a silica gel (Merck 70 - 230 mesh) column eluted with acetone-water (5:1). Fractions (15ml) were monitored for cyanoglycoside using cerric(IV) sulphate reagent and an enzymic procedure as described in chapter 1. The fractions were thus grouped into the following samples according to their cyanogenic properties:

Sample	Fractions	Properties
1A	1 to 35	Cyanogenic
1B	36 to 65	Transitional (Weakly cyanogenic)
1C	66 to 120	No cyanoglycoside

These samples were evaporated to syrups using a rotary vacuum evaporator, weighed, and redissolved in methanol to concentration 40mg/ml. A sample of crude extract (before chromatography) was prepared in the same way (= sample 1D). Filter paper discs (Whatman No. 1, 1.8cm) were soaked in each of these solutions for approximately 10 seconds and the solvent was evaporated by placing the discs in an oven at 40°C for 20 minutes. In addition, a control soaked in pure methanol was treated in the same way.

Six replicate sets of discs were prepared in this way, and five discs of each set (i.e. samples 1A-D and the control) were attached to the walls of a separate plastic container using thin strips of masking tape. Five fifth instar *A. horta* larvae were added to each container, the paper discs moistened with distilled water, and the containers were closed. The experiment was allowed to run for 48 hours, during which time the paper discs were re-moistened three times. Details of collection and treatment of caterpillars prior to the experiment, growth chamber conditions and the containers used were identical to those described above for the leaf feeding experiment.

The amount of each filter paper disc eaten by larvae was measured by carefully cutting around the edge of photocopied versions of the partially eaten discs. The ratio of the mass of each of these to the mass of an uneaten disc treated in the same way, expressed as a percentage, gave an index of palatability of the samples.

Experimental details of phase 2 and phase 3 below were almost identical to those described above. In what follows only the important points in which the phases differed are described.

Phase 2: An extract of *K. africana* leaves was eluted from the column using two progressively more polar solvent systems in order to effect a clear separation between the cyanoglycoside and the more polar molecules to which caterpillars had responded in phase 1. The first of these solvent systems was chloroform-methanol (5:2), from which the following samples were recognised:

Sample	Fractions	Properties
2A	1 to 40	No Cyanoglycosides
2B	41 to 90	Cyanogenic
2C	91 to 160	Transitional (Weakly Cyanogenic)

The column was then allowed to run dry, and re-eluted using acetone-water (5:1, 1500ml), to yield sample 2D which showed no cyanogenic activity. A sample of crude extract (= sample 2E) and a control dipped only in methanol were again included. In phases 2 and 3, unlike phase 1, only five experimental replicates were included.

Phase 3: This phase of the experiment was designed to test whether the feeding response of *A. horta* larvae in phases 1 and 2 was due to the presence of primary metabolites (e.g. sugars or amino acids) common to all plants, or to some secondary substance of limited distribution among plants. *Homalium dentatum* (Harvey) Warb. (Flacourtiaceae) and lettuce were selected as species assumed to contain no chemical deterrents to insect feeding. For lettuce, this assumption was based of the non-bitter taste of the young succulent leaves used in the experiment. Taste is a good test for the presence of deterrent substances in leaves since virtually all biological toxins are bitter tasting to humans (Brower, 1984). *H. dentatum* has tough leathery leaves, with low water content and a serrated leaf edge and is therefore morphologically unsuited to insect herbivory. It was thus assumed to be a likely species lacking chemical defences and this might account for the absence of cyanoglycosides, the toxins produced by several members of the Flacourtiaceae, in this species (Table 1). These assumptions were partially confirmed since both lettuce and *H. dentatum* were found to lack alkaloids using the spot test of Raffauf (1962). Furthermore, lettuce was found to contain low levels of polyphenols relative to *K. africana*, and levels in *K. africana* and *H. dentatum* were roughly equal (Folin-Denis test; Allen, 1974).

Crude extracts of *K. africana*, lettuce leaves, and *H. dentatum* were prepared. These were made up to concentrations equal to 80 and 160mg/ml (lettuce and *H. dentatum*) and 40, 80 and 160mg/ml (*K. africana*) and offered to fifth instar larvae as described above.

RESULTS

Leaf Palatability Experiment

Table 1 shows the plant species included in each stage of the experiment, the amount of leaf eaten by larvae, and whether each plant species was cyanogenic or not. A significant relationship ($p<0,005$; Fisher Exact Probability Test) between presence of cyanoglycoside in leaves and accceptance by larvae existed over all species tested and this relationship was also significant ($p<0,05$) for cyanogenic species in the Flacourtiaceae alone. It was not possible to test this for Passifloraceae alone, since no acyanogenic species in this family were available. Caterpillars offered *Adenia hastata* and *A. digitata* showed signs of poisoning as they adopted a characteristic contorted body position on contact with the leaves.

Artificial Feeding Experiment

Phase 1: Figure 1 shows that *A. horta* larvae responded to fractions of *K. africana* leaf extract that contained cyanoglycoside (samples 1A and 1B) and those that did not (sample 1C). There was a significantly greater response ($p<0,025$; Student-t test) to the partially purified sample 1C than to the unpurified leaf extract (sample 1D).

Phase 2: Larvae showed barely any response to fractions of *K. africana* extract eluted from the chromatographic column with chloroform-methanol (5:2) and these included all fractions containing cyanoglycoside (samples 2B and 2C) (Figure 2). There was, however, a strong response to fractions eluted with the more polar solvent system acetone-water (5:1) (sample 2D), none of which contained cyanoglycoside. As in phase 1 of the experiment, there was a significantly larger ($p<0,005$) response to the partially purified fractions (sample 2D) than to crude extract (sample 2E).

Phase 3: Figure 3 shows that larvae did not respond to extracts of lettuce or *H. dentatum* leaves at 80 and 160mg/ml, or to *K. africana* at 40mg/ml. They did however, respond to *K. africana* extract at 80 and 160mg/ml, but there was no significant difference between the levels of response to these ($p>0,95$).

TABLE 1. Edibility of twelve cyanogenic and eight acyanogenic species of Passifloraceae (P) and Flacourtiaceae (F) to fifth instar *Acraea horta* larvae. The data were collected in four stages (left hand column). Edibility is expressed as the percentage of the weight eaten (g/g of caterpillar/24 hrs) of a *Kiggelaria africana* control included in each stage.

Stage	Species	Family	HCN	Edibility $\bar{X}$ (N=5)	S.D.
1	*K. Africana* (control)	F	+	0.55 g/g/24hr	0.09
	Adenia repanda	P	+	10.4%	5.4
	Adenia fruticosa	P	+	26.1%	10.9
	Xylotheca krausiana	F	+	16.8%	13.3
	Rawsonia lucida	F	+	4.9%	4.0
	Trimeria grandifolia	F	-	Not eaten	
	Scolopia mundii	F	-	Not eaten	
	Adenia hastata	P	+	Not eaten	
	Flacourtia indica	F	-	Not eaten	
2	*K. africana* (control)	F	+	0.56 g/g/24hr	0.04
	Adenia glauca	P	+	8.3%	4.7
	Passiflora edulis	P	+	5.4%	4.3
	Passiflora caerulea	P	+	94.3%	20.1
	Dovyalis caffra	F	-	Not eaten	
	Homalium dentatum	F	-	Not eaten	
	Adenia digitata	P	+	Not eaten	
3	*K. africana* (control)	F	+	0.49 g/g/24hr	0.13
	Tacsonia mollissima	P	+	30.6%	11.1
	Dovyalis spinosa	F	-	Not eaten	
	Flacourtia sp.	F	-	Not eaten	
	Dovyalis rhamnoides	F	-	Not eaten	
4	*K. africana* (control)	F	+	0.64 g/g/24hr	0.11
	Tacsonia manicata	P	+	100%	17.4

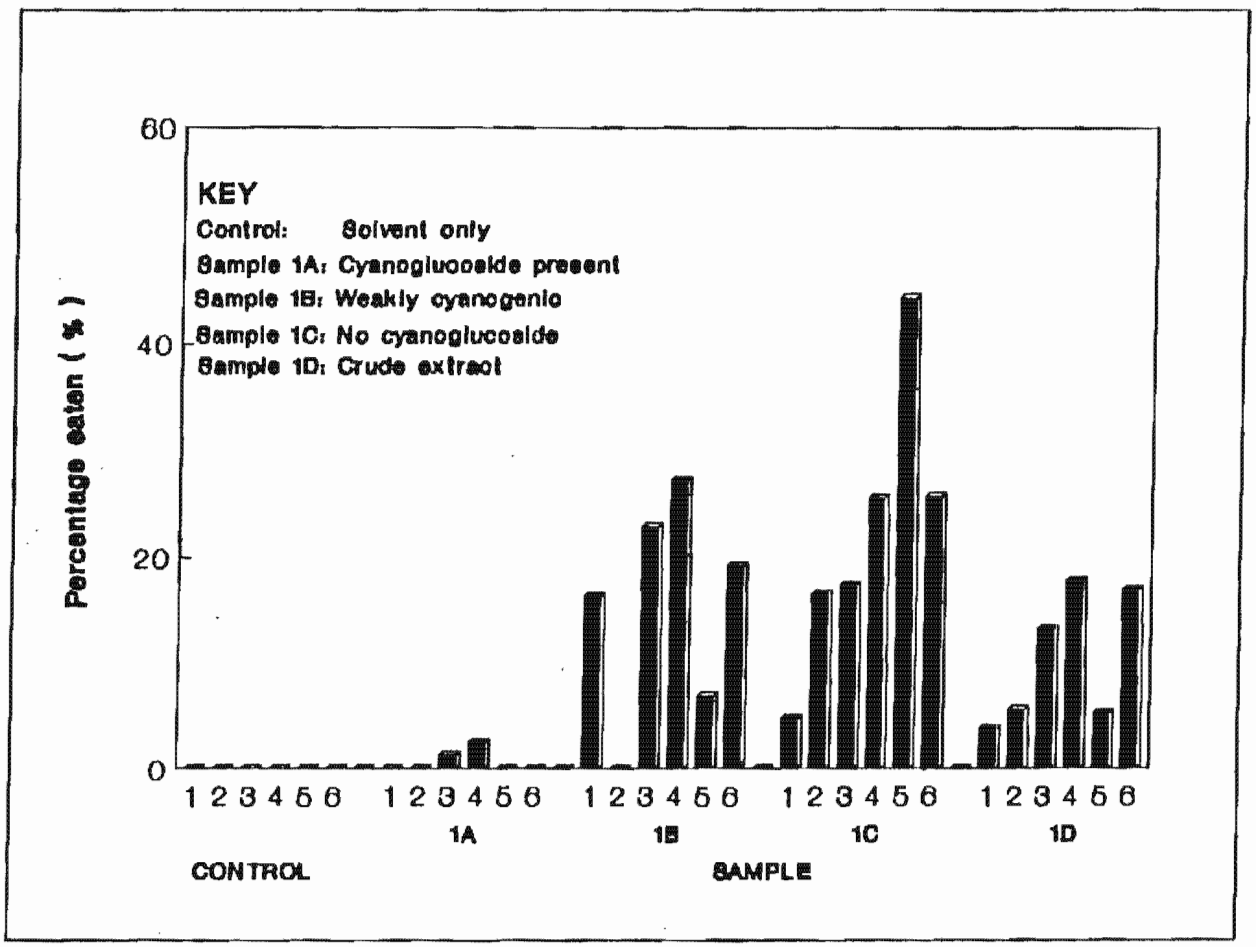


FIGURE 1. Feeding response of *A. horta* larvae to paper discs dipped in samples of *K. africana* leaf extract eluted from a chromatographic column with acetone-water (5:1). Nos 1-6 represent experimental replicates of treatments A-D.

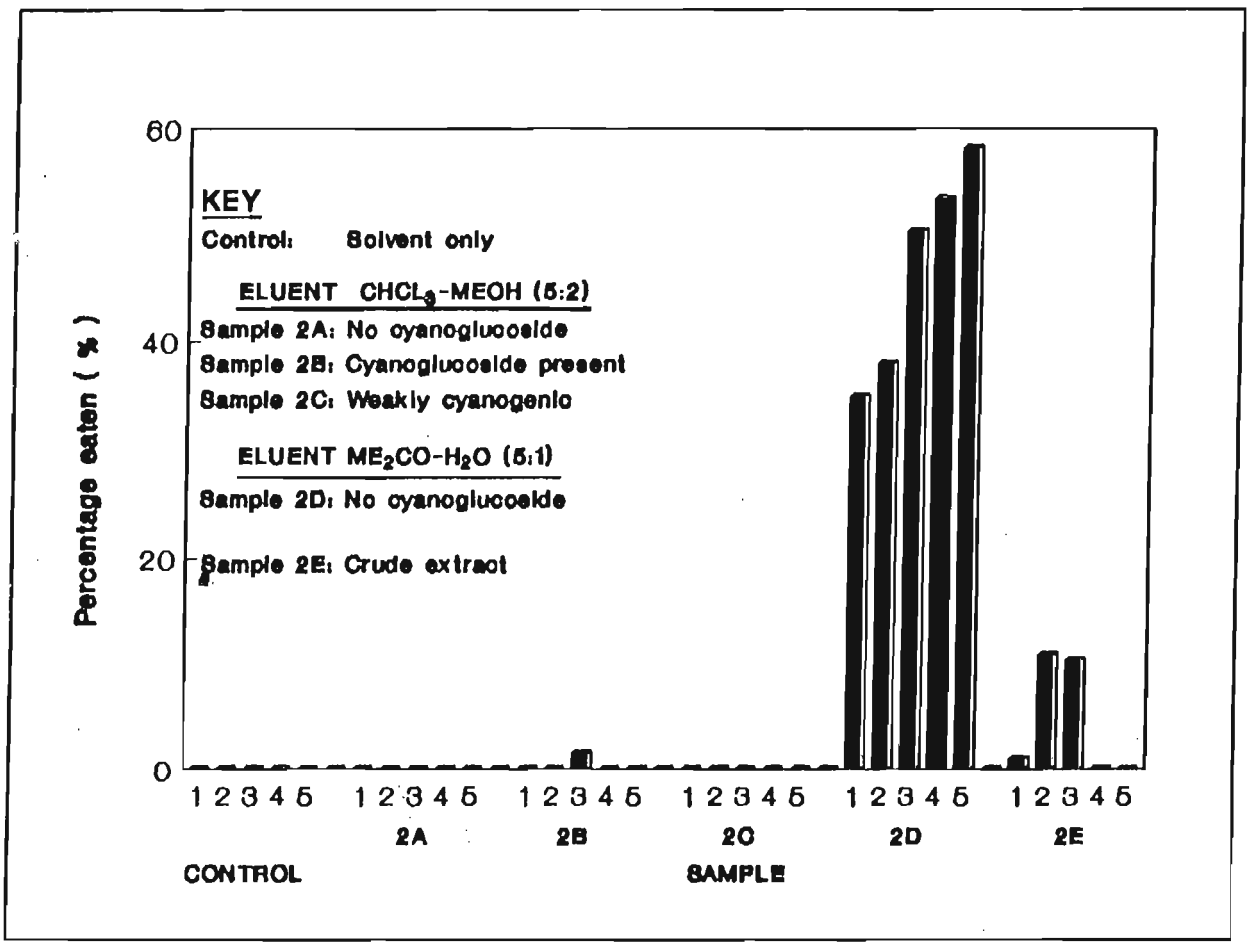


FIGURE 2. Feeding response of *A. horta* larvae to paper discs dipped in samples of *K. africana* extract eluted from a chromatographic column with progressively more polar solvent systems. Nos 1-5 represent experimental replicates of treatments A-E.

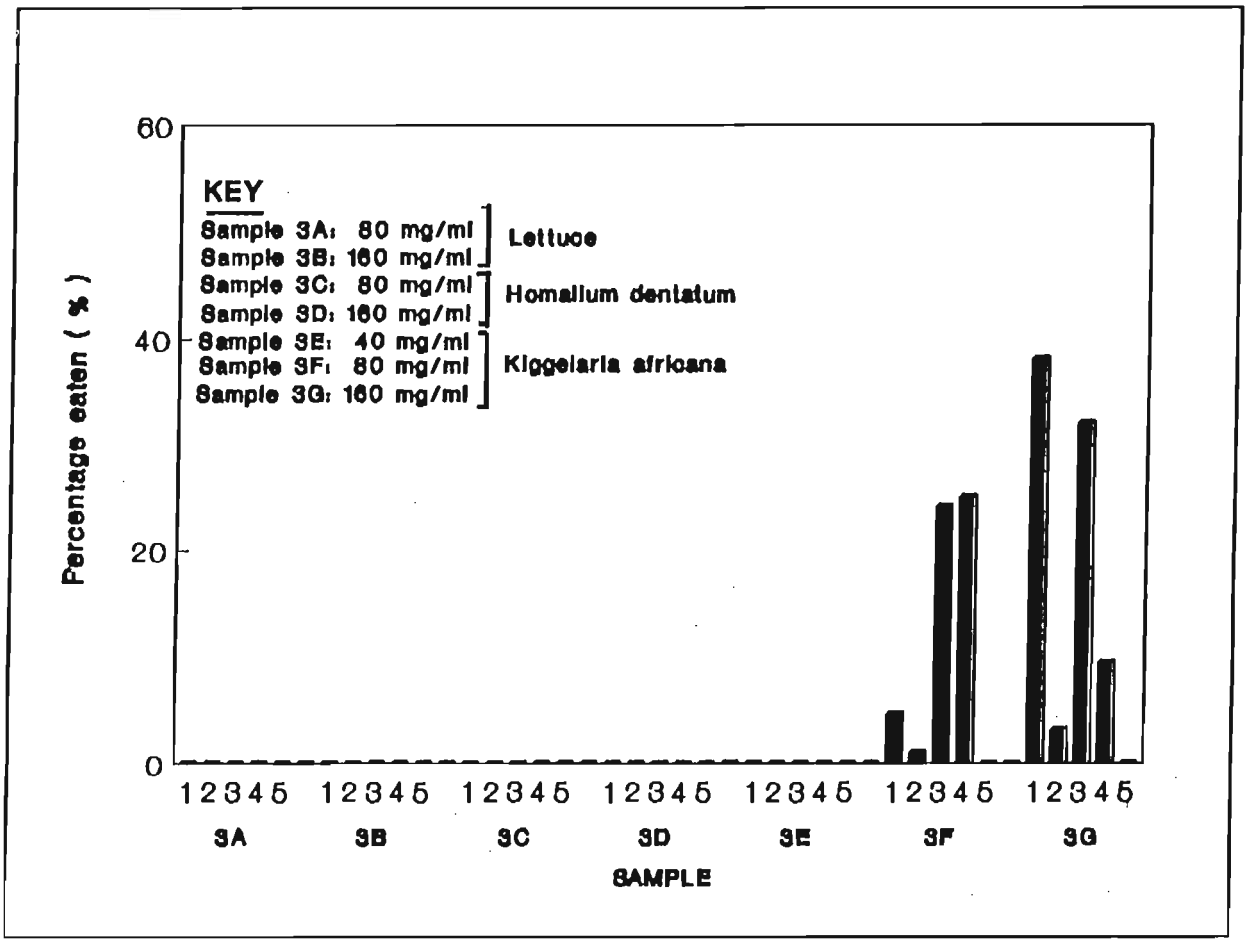


FIGURE 3. Feeding response of *A. horta* larvae to paper discs dipped in ethanolic extracts of the leaves of lettuce and *Homalium dentatum* at two concentrations each, and *K. africana* at three concentrations. Nos 1-5 represent experimental replicates of treatments A-G.

DISCUSSION

My results suggest that fifth instar *A. horta* larvae are capable of selecting cyanogenic species from among potential food plants in the Flacourtiaceae and Passifloraceae. A positive relationship between acceptance and the presence of cyanoglycosides in leaves existed when plants in both families were treated as a single group, and among plants in the Flacourtiaceae alone. All species in the Passifloraceae were cyanogenic and all, except *Adenia digitata* and *A. hastata*, were eaten by larvae. Caterpillars exposed to the latter two species showed signs of poisoning on mere contact with the leaves and it is likely that rejection was due to the presence of a second toxin to which the insects are susceptible. One possible cause is modeccin, a potent ribosome-inhibitor which has been detected in *A. digitata* (Watt and Breyer-Brandwijk, 1962). It is also known that several species of Passifloraceae produce toxic alkaloids (Willaman and Schubert, 1961). It is therefore likely that, in the absence of inhibitory substances, *A. horta* larvae select cyanogenic species in the Passifloraceae as well. This conclusion should, however, be treated with caution until acyanogenic Passifloraceae are available for inclusion in such a test, although it is possible that no such species will be found (Gibbs, 1965).

Results of the artificial feeding experiment suggest that the selective feeding response of *A. horta* larvae to cyanogenic leaves may not be based on the use of cyanoglycosides as a feeding stimulant. There are, however, considerable complexities involved in investigating the factors responsible for food choice by phytophagous insects, and in this regard the power of artificial feeding experiments of the type described here is limited. Where there is good reason to believe that a specific feeding stimulant is responsible for host choice (e.g. glucosinolates and *Pieris brassicae* - Verschaffelt, 1910), such tests are appropriate. If the insect responds only to fractions containing the postulated feeding stimulant, this provides strong evidence that this compound plays an important role in determining host specificity. However, if the insect does not respond to the compound under investigation, the results are more difficult to interpret. Some secondary compounds may only stimulate feeding in the presence of particular primary metabolites, and there may be concentration thresholds beyond which feeding stimulants have a repellent effect. For example, Nayar and Fraenkel (1963) found that the mexican bean beetle, *Epilachna varivestis* (Mulsant), is stimulated to feed by the cyanoglycosides linamarin and lotaustralin, but only in the presence of glucose. Futhermore, at concentrations higher than those

usually found in the food plant of these beetles, the cyanoglycosides are toxic and actually inhibit feeding.

In the present experiment *A. horta* larvae ate both cyanogenic and acyanogenic fractions (Figure 1), and when the cyanoglycoside was first removed using a less polar solvent system they only ate the acyanogenic fractions which remained on the column until eluted with the more polar acetone-water (5:1) (Figure 2). These data do not prove that gynocardin is not a stimulant to feeding by *A. horta* larvae, since the concentrations of this substance in samples 1A, 2B and 2C may have been above those normally encountered in plants, thus inhibiting feeding by the insects (e.g. Nayar and Fraenkel, 1963). Similarly, the possibility that gynocardin only stimulates feeding synergistically in the presence of, for example, sugars was not investigated. Simple sugars, such as glucose, are more polar than gynocardin and would thus indeed have been separated from gynocardin during chromatography. It is, however, unlikely that gynocardin stimulates feeding synergistically with sugars or other primary metabolites, since larvae responded more strongly to acyanogenic fractions (samples 1C and 2D) than to crude extracts containing both primary metabolites and cyanoglycoside (samples 1D and 2E). Moreover, the presence of gynocardin in sample 1B did not enhance the feeding response relative to sample 1C which did not contain this compound.

My data do, however, prove that gynocardin is not an *essential* feeding stimulant to *A. horta* larvae. That is, under the circumstances of the experiment at least, some other substance (or combination of substances), which is more polar than gynocardin, occurs in the leaves of *K. africana* and stimulates feeding by *A. horta* larvae. This substance could be a primary metabolite (e.g. sugars, amino acids, minerals, vitamins, organic acids - all of which are known to stimulate feeding in some insects - Thorsteinson, 1960), or a specific secondary compound of limited distribution among plants. If a primary metabolite, then larvae should respond equally to the tissues of other plants lacking specific deterrent substances, since all green plants are believed to contain essentially the same primary metabolites (Fraenkel, 1969). This was, however, not the case, since *A. horta* larvae rejected extracts of the leaves of both lettuce and *Homalium dentatum*, while feeding on equivalent extracts of

K. africana (Figure 3). I therefore suggest that a specific substance (or combination of substances) of limited distribution among plants, and which co-occurs with cyanoglycosides in the leaves of Passifloraceae and Flacourtiaceae, stimulates feeding by *A. horta* larvae. This conclusion, however, rests on the assumption that feeding deterrents undetectable by the tests performed did not exist in the leaves of both *H. dentatum* and lettuce.

A quantitative relationship should exist between the concentration of a feeding stimulant and the feeding response of insects (Fraenkel, 1959), and there is some evidence of this in the present study. Partially purified samples and crude extracts in each respective phase were of approximately the same concentration, and dilution would result in the feeding stimulant therefore being less concentrated in crude extracts. Larvae should thus respond more strongly to the partially purified extracts, and Figures 1 and 2 show that this was indeed the case in both phases of the experiment. Also, larvae did not respond to crude extract at 40mg/ml in phase 3, but readily ate those at 80 and 160mg/ml (Figure 3). There was, however, no significant difference between the response to the latter two samples.

It may be expected, in view of the evidence that *A. horta* obtains cyanoglycosides from the leaves they eat (Chapter 2), that these compounds would play a role in host selection by these insects. However, evidence presented here suggests that the situation for *A. horta vis á vis* cyanoglycosides may be different from that of *Pieris brassicae vis á vis* the glucosinolates produced by their cruciferous food plants. The interesting possibility exists that *A. horta* larvae are able to select cyanogenic hosts by responding to a substance that predictably co-occurs in leaves together with cyanoglycosides. Dethier (1954) cited evidence suggesting that phytophagous insects use nutritionally unimportant token stimuli to recognise plants containing the balance of nutrients to which they are adapted. He later (1972) extended this concept, suggesting that insects may use token stimuli to recognise plants containing toxic substances from which the insects derive secondary advantages. This has, as far as I know, not hitherto been demonstrated. The present study may therefore be the first example of the use by insects of a token stimulus to recognise the presence in plants of toxic substances they sequester for defensive purposes. This hypothesis is currently based on circumstantial evidence which has yet to be corroborated by the isolation and identification of the substance (or substances) that stimulate feeding by this species on cyanogenic plants.

GENERAL CONCLUSIONS AND SOME DIRECTIONS FOR FURTHER RESEARCH

The research reported here has established *A. horta* as an important hitherto 'missing link' in our understanding of cyanogenic Lepidoptera. In the face of this new evidence it now seems unlikely that the ability of cyanogenic Lepidoptera to synthesise cyanoglycosides preceded their association with cyanogenic plants. Rather, it is more parsimonious to suggest that the colonisation of cyanoglycoside-producing plants by these insects lead to their ability to synthesise these toxins and hence, to expand their host range while retaining an aposematic way of life. Existing patterns of host utilisation suggest that this process of diversification may still be in progress and it would be interesting to establish the relationship between choice of host plants and cyanide metabolism for a wide range of Acraeinae. Perhaps some acraeine species - like *Zygaena trifolii* - will be found to both sequester and synthesise cyanoglycosides, thus representing an evolutionary stage intermediate between those that sequester (e.g. *A. horta*) and those known to synthesise these compounds (e.g. *A. encedon*). A detailed comparison of these data with other, systematic (e.g. morphological) criteria may go a long way towards elucidating the evolutionary processes accounting for the patterns of host utilisation by Acraeinae.

Similar evolutionary processes may account for the evolution of patterns of host choice among *Heliconius* butterflies. This would, however, be more difficult to demonstrate since no *Heliconius* species are known to feed on acyanogenic plants, and only a few feed outside of Passifloraceae. Those that do, feed on the closely related Turneraceae (Smiley, 1985). Heliconiine butterflies have close ecological adaptations to their *Passiflora* host plants (Turner, 1981) and it is therefore possible that these insects, while having lost the ability to sequester and having evolved the means to synthesise cyanoglycosides, have not had the opportunity to broaden their host range (Feeny, 1975). It would be interesting to look among primitive members of this group for species that contain cyclopentenyl cyanoglycosides, as do their food plants. It is, however, possible that no extant heliconiine species will be found to sequester cyanoglycosides, since Nahrstedt and Davis (1983) found linamarin and lotaustralin in members of seven genera of these butterflies.

The present study considered only qualitative aspects of cyanoglycosides in *A. horta*. The variation of gynocardin in various stages of the life cycle, and the rates of

transfer of this compound between these stages now need to be determined. Also, the possibility that these organisms synthesise gynocardin in addition to obtaining this compound from plants needs to be further investigated. It would also be interesting to determine whether the larvae are capable of sequestering linamarin and lotaustralin and whether, given a dietary source of free valine and isoleucine, these insects could synthesise these cyanoglycosides (A. Nahrstedt, personal communication). Such aspects have been investigated for *Heliconius melpomene* (Nahrstedt and Davis, 1983), *Zygaena filipendulae* (Wray *et al.*, 1983) and *Z. trifolii* (Nahrstedt and Davis, 1986), and the radioisotope techniques employed in these studies would be well suited to furthering research on *A. horta*.

The discovery that *A. horta* larvae respond to a feeding stimulant other than cyanoglycosides and are still capable of selecting cyanogenic species from among potential food plants needs further investigation, including electrophysiological and further behavioural studies, as well as detailed chemical analyses. Should the results of such investigations corroborate the findings of the present study, then some interesting questions arise regarding host selection by *A. horta*. For example, why do these insects not respond to the cyanoglycoside itself? The answer to this may be historical, or it may be that the feeding stimulant has some favourable chemical property, such as being more exposed to the chemoreceptors than are cyanoglycosides. The feeding stimulant may be a precursor, or an intermediate, in the synthesis of cyclopentenyl cyanoglycosides and would thus be a reliable token indicating the presence of these secondary compounds in plants. Comparison with similar aspects of the feeding specificity of species closely related to *A. horta* may provide valuable information regarding the evolution of acraeine feeding patterns. The chemical basis of host selection by adult females also needs detailed consideration and it would be interesting to determine whether the oviposition stimulant to these insects is the same substance as the larval feeding stimulant.

Finally, the present study has revealed a potential means of researching the defensive role of cyanoglycosides in insects. Jones (1971) suggested that a defensive function can only be established for a secondary compound if the reaction of potential predators to toxic and non-toxic members of the same species can be determined. This aspect can be taken further by quantifying the response of predators (e.g. Brower and Brower, 1964) to organisms containing variable amounts of the putative toxin. Such comparisons were not hitherto possible for cyanogenic insects, but the discovery

that *A. horta*, containing little or no cyanoglycoside, can be reared on *Tacsonia mollissima* (chapter 2) may now make this possible. Imagines containing variable amounts of cyanoglycoside may be reared by transferring the larvae from cyanogenic to acyanogenic plants at different stages of their development. It was, however, difficult to rear *A. horta* on *T. mollissima* and this aspect therefore needs further attention. Nonetheless, another potential means of producing imagines containing variable concentrations of cyanoglycosides has arisen from this study. Since *A. horta* larvae respond to a feeding stimulant which is itself not cyanogenic, it should be possible to induce these insects to feed on acyanogenic food. In chapter 3 it was shown that larvae would indeed feed on filter paper soaked in this substance, and it may be possible to rear these insects on the leaves of acyanogenic plants in this way. Alternatively, artificial diet containing the feeding stimulant together with variable amounts of artificially added cyanoglycoside could be fed to larvae.

A. horta has therefore proved to be an interesting organism for the study of cyanogenic Lepidoptera. Indeed, further exploration of the discoveries reported here could elucidate causes of the distinct patterns of host utilisation by cyanogenic Lepidoptera and thereby contribute to an overall understanding of the three areas of unusual diversity in biology: the plants, the insects and plant secondary compounds.

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