Comp. Biochem. Physiol. Vol. 75B, No. 1, pp. 65 to 73, 1983
Printed in Great Britain

0305-0491/83/050065-09$03.00/0

OCCURRENCE, VARIATION AND BIOSYNTHESIS OF THE CYANOGENIC GLUCOSIDES LINAMARIN AND LOTAUSTRALIN IN SPECIES OF THE *HELICONIINI* (INSECTA: LEPIDOPTERA)

A. NAHRSTEDT
Institut für Pharmazeutische Biologie, Technische Universität, D-3300 Braunschweig, FRG
and
R. H. DAVIS
Wye College, University of London, Wye, Ashford, Kent TN25 5AH, UK

(*Received* 16 *September* 1982)

Abstract—1. Cyanogenesis has been confirmed in all neo-tropical genera of the tribe *Heliconiini* and in the tropical genus *Cethosia*.

2. In all cyanogenic species the source of cyanide is the glucosides linamarin and lotaustralin, the former always being present in the greater amount.

3. All stages of the life cycle of *Heliconius* itself are cyanogenic, with the highest concentration of cyanide in ova and higher concentrations in females than in males.

4. Experiments with isotopes have shown that the two cyanoglucosides are synthesized by *Heliconius* from the amino acids valine and isoleucine at both larval and adult stages.

5. The evidence suggests that both catabolism and anabolism of cyanoglucosides occurs during the pupal stage.

INTRODUCTION

Heliconiini, a neo-tropical tribe of the *Nymphalidae*, have been shown to possess distasteful properties to both birds (Brower *et al.*, 1963) and lizards (Boyden, 1976). Furthermore, extracts prepared from imagines of *Heliconius erato* were found to be lethal when injected intraperitoneally into mice although extracts from two other *Heliconius* species had no observable effect (Marsh & Rothschild, 1974). There appears to be no published evidence on the chemical basis of the distasteful properties of these butterflies and the only observation that might relate to their potential toxicity was the finding that adults contain cyanoglucosides within their tissues (Nahrstedt & Davis, 1981a). In that study two cyanoglucosides, linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile) and lotaustralin (2-β-D-glucopyranosyloxy-2-methyl-2R-butyronitrile) were identified in an extract prepared from *H. erato* and *H. doris*.

The larval foodplants of *Heliconiini*, species of *Passifloraceae*, are also cyanogenic but the cyanoglucosides most associated with this plant family are different, containing cyclopentenyl moieties and hence structurally and biogenetically unrelated (Tantisewie *et al.*, 1969; Conn, 1980).

There has been a recent report that some *Passiflora* spp. contain other cyanoglucosides, including linamarin (Fung *et al.*, 1981; Fischer *et al.*, 1982) but larvae obviously are not dependent upon a dietary supply since they are able to synthesize linamarin and lotaustralin themselves (Nahrstedt & Davis, 1981b).

In this report further information is given on the occurrence of cyanoglucosides in *Heliconius* and related genera, with results for all stages of the life cycle of particular species. Initial results on the biosynthesis of the glucosides are included also. This new information is discussed in relation to current views on the taxonomy of these genera and to particular aspects of their biology and biochemistry.

MATERIALS AND METHODS

Heliconius melpomone and *H. charitonia* were reared on *Passiflora coerulea*, except for studies on the influence of larval foodplant in which different species of *Passiflora* were used, as indicated. The other heliconian butterfly species were obtained from the British Museum (London) via Dr M. Boppré (formerly Seewiesen). *Cethosia biblis*, *C. hypsea*, *Acraea violae* and *Vindula arsinoe* were provided by Worldwide Butterflies, Sherborne, Dorset; *C. hypsea* and *Parthenos sylvia* by Universiti Pertanian Malaysia, Serdang, Selangor; and *A. andromachae* by University of Queensland, St. Lucia, Queensland; all as air dried specimens with the exception of *A. violae* which were received as live pupae.

L-[U-^{14}C]valine and L-[U-^{14}C]isoleucine were purchased from The Radiochemical Centre, Amersham, UK.

Application of amino acids

Valine and isoleucine were administered to larvae either with their food or by direct injection into the haemocoele. When oral dosage was used the methanolic solution of amino acid(s), containing the minimum quantity of HCl necessary to achieve solution at the required concentration, was applied to a small portion of leaf and the solvent

allowed to evaporate. Treated leaf was offered to larvae once or twice daily depending upon the rate at which the offered food was consumed. At all other times larvae had continuous access to food. This procedure was used for all experiments involving normal valine and isoleucine. For those experiments involving ^{14}C amino acids, the amino acids were either given in the same way or by the injection of 10 μl of aqueous solution, that provided 0.25 μCi of activity per amino acid to each larva.

The amino acids were fed to imagines by allowing them free access to a solution of amino acid, 0.1 M in 10% sucrose, for a period of at least 2 weeks. Imagines were randomly allocated either to the amino acid treatment or to a 10% sucrose control treatment as soon after eclosion as possible and the 2-week feeding period commenced as soon as the full complement had been obtained. In the experiment in which ^{14}C amino acids were fed to imagines a 15 μl drop of an aqueous solution containing 0.25 μCi from both valine and isoleucine together with glucose was placed on a watch glass. The proboscis was uncoiled with the aid of a needle and the tip held in the solution. In this way the labelled amino acids were fed to both freshly emerged males or to females taken immediately after oviposition thus ensuring that all females were mature egg-layers.

Estimation of cyanoglucosides

The procedures for extraction of glucosides, their separation and identification as TMSi-ethers by gas–liquid chromatography and the quantitative estimations of cyanide were the same as were described previously (Davis & Nahrstedt, 1979; Nahrstedt & Davis, 1981a). In all tables the amount of cyanide enzymatically liberated from linamarin and lotaustralin represents the total content of cyanoglucosides.

Estimation of radioactivity

The radioactivity incorporated into linamarin and lotaustralin was estimated as described in Fig. 1. The total glucoside content was measured from the MeOH/EtOAc extract as described earlier (Davis & Nahrstedt, 1979; Nahrstedt & Davis, 1981a) using a linamarase isolated from *Hevea brasiliensis* with high activity on linamarin and lotaustralin (Selmar, 1981). The quantitative ratio of linamarin:lotaustralin was estimated by GLC (Davis & Nahrstedt, 1979). Both glucosides, linamarin and lotaustralin were separated by HPLC (Davis & Nahrstedt, 1979), and the radioactivity counted in a Betazint BF 5000 (Berthold, Wildbad) using Unisolve 100 (Zinsser, Frankfurt) as a scintillation cocktail. Each glucoside was then hydrolysed using the linamarase mentioned above and the cyanide trapped was estimated quantitatively (Nahrstedt, 1977). Additionally the radioactivity of trapped cyanide was measured using the same cocktail as above. Normally the ratio of activity of linamarin to cyanide liberated from linamarin was in the range of 4:1, and that of lotaustralin to cyanide isolated from lotaustralin was in the range of 5:1. Because of its higher accuracy the specific radioactivity of linamarin was calculated by multiplication of the activity of its cyanide by four and that of lotaustralin by multiplication of the activity of its cyanide by five. This procedure is allowed as incorporation with U-[^{13}C]valine and U-[^{13}C]isoleucine showed, that the total amino acid except the carboxyl group is incorporated without disruption of carbon bonds into both glucosides (Wray *et al.*, in preparation).

In some experiments (e.g. Table 7) the radioactivity of the defatting petrol extract and the methanolic extract containing the cyanoglucosides as well as the activity which remained in the insoluble residue was estimated. For the latter the residue was hydrolysed by 1 N HCl, but in every case total solubilization did not occur, thus resulting in a total percentage less than 100% relative to the dosed total radioactivity. However, it is obvious that most of the amino acids are used for the synthesis of body building substances.

RESULTS

Distribution of linamarin and lotaustralin

Air-dried specimens of six genera of heliconiines, other than *Heliconius* itself, were available for testing and all were found to be cyanogenic. So too were two species of *Cethosia* and two of *Acraea* (Table 1). However, *Parthenos sylvia* and *Vindula arsinoe* both nym-

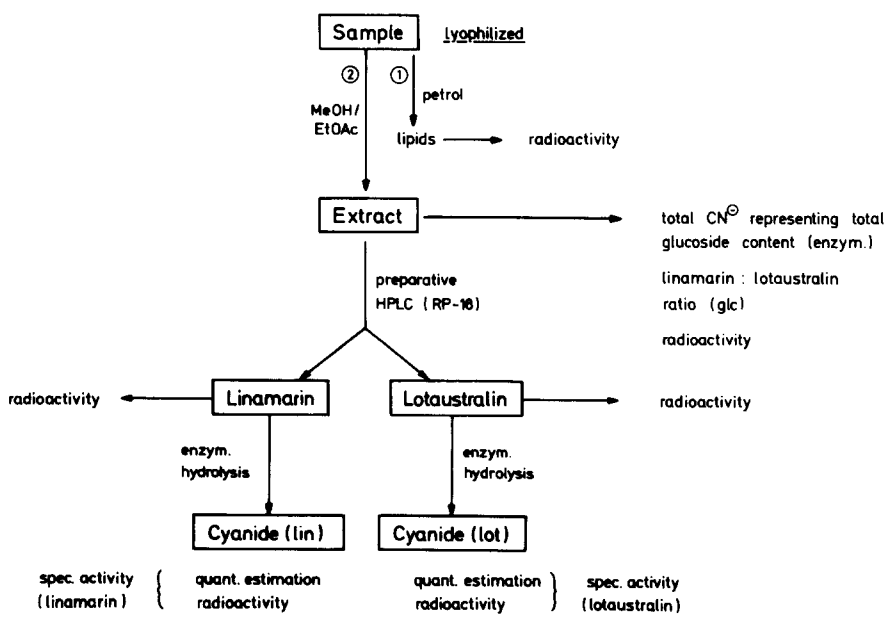

Fig. 1.

Table 1. The quantity of cyanide and proportions linamarin and lotaustralin found in seven species of *Heliconiini*, two of *Cethosia* and two of *Acraea*

Species	Number analysed	Dry matter (mg)	μmol/g/dm	CN^- per individual	Ratio linamarin: lotaustralin
Dryas julia	2	74	0.9	0.03	80:20
Dryadula phaetusa	2	135	1.4	0.1	93:7
Agraulis vanillae	2	149	4.4	0.32	93:7
Dione juno	2	91	0.08	0.004	+ n.d.
Podotricha telesiphe	1	40	0.2	0.008	+ n.d.
Podotricha euchroia	1	30	1.6	0.05	+ n.d.
Philaethria dido	2	150	0.4	0.03	80:20
Cethosia hypsea	2	163	10.8	0.88	97:3
Cethosia biblis	3	245	0.94	0.08	+ n.d.
Acraea andromache	12	394	1.67	0.055	n.d. n.d.
Acraea violae	11	369	43	1.45	+ + + n.d.
Acraea violae pupae	5	200	48	1.92	+ + + n.d.

n.d., not detectable with certainty from glc signal.

phalids that also feed upon *Passifloraceae* (Corbet & Pendlebury, 1978), did not contain linamarin and lotaustralin. In all cases in which the proportions of linamarin and lotaustralin could be determined the former was present in the larger amount.

Variation of cyanoglucosides during life cycle

Analyses performed on fresh material from all stages of the life cycle for *H. charitonia* showed that there was a particularly high concentration of cyanoglucosides in eggs on weight basis (Table 2). The content decreased after pupation on an individual basis but the decrease was not in proportion to the decrease in body weight and so the concentration of cyanoglucosides in imagines was higher than in either larvae or pupae. There was more linamarin present than lotaustralin at all stages and a consistent increase in the proportion of linamarin during develop-

Table 2. The quantity of cyanide and proportion of linamarin and lotaustralin determined at different stages of the life cycle of *H. charitonia* and *H. melpomone*

Sample	Number analysed	Dry matter (mg)	μmol CN^- per gdm	μmol CN^- per individual	Ratio linamarin: lotaustralin
H. charitonia					
Larvae	37	2877	34.7	2.70	66:34
Pupae	25	1707	31.5	2.15	79:21
Imagines ♂	17	644	50.8	1.92	77:23
Imagines ♀	22	813	56.8	2.10	80:20
Eggs	20	4	171.7	0.04	63:37
H. melpomone					
Larvae	6	268	39.8	1.78	90:10
Imagines ♂	23	496	61.1	1.32	88:12
Imagines ♀	10	225	64.1	1.44	85:15

Table 3. The quantity of cyanide and proportions of linamarin and lotaustralin present in the late larval and pupal stages in *H. melpomone*

Sample	Number analysed	Dry matter (mg)	μmol CN^- per gdm	μmol CN^- per individual	Ratio linamarin: lotaustralin
Larvae					
Still feeding	6	494	14.0	1.15	91:9
Ceased feeding	6	570	10.8	1.02	93:7
Suspended	6	385	16.0	1.02	94:6
Pupae					
1st day ♂	4	348	14.9	1.30	95:5
1st day ♀	4	271	22.0	1.50	95:5
4th day ♂	4	285	10.8	0.77	96:4
4th day ♀	4	221	23.5	1.30	95:5

Table 4. The quantity of cyanide and proportions of linamarin and lotaustralin found in different parts of *H. charitonia* larvae*

Sample	Dry matter (mg)	μmol CN^- per gdm	μmol CN^- per individual	Ratio linamarin: lotaustralin	(%) of total† CN^-
Haemolymph	51.4	47	0.24	61:39	29
Fat body	30.5	23.6	0.07	64:36	8
Gut	113	2.2	0.025	~78: ~22	3
Muscles and skin	247	20.3	0.50	67:33	60
Summation	442		0.835		100

* Ten larvae were dissected in 0.75% saline.
† Assuming total dissection and recovery of cyanide.

ment is evident, until pupation at least. For *H. melpomone* total glucoside (represented by cyanide) concentration was also higher in imagines than larvae and the ratio of linamarin to lotaustralin was higher than in *H. charitonia* for comparable samples. The amount and concentration of cyanoglucosides tended to be higher in female imagines than in males for both species.

In Table 3 results from a more detailed study of the changes occurring during the late larval and pupal stages of *H. melpomone* are given. These show that there is a difference in cyanoglucoside concentration between the sexes from the first day of the pupal stage. There was also a consistent increase in the amount of linamarin relative to lotaustralin with progression in development which was independent of sex. It is possible that a sex difference in cyanoglucoside content and concentration exists at the larval stage but this was not determined.

Distribution of cyanoglucosides in larvae

Analyses of larvae that had been crudely dissected into 3 parts following the withdrawal of haemolymph show that the greatest amount of cyanoglucosides is to be found in the muscles and skin and that most of the remainder is present in haemolymph (Table 4). The amounts found in the gut and fat body were small and may have resulted either from incomplete removal of haemolymph or from incomplete separation during dissection. The proportion of linamarin was lower in haemolymph than in muscle and skin.

Influence of food on cyanoglucoside content

The influence of larval foodplant on cyanoglucosides in *H. melpomone* was studied using eight different species of *Passiflora* (Table 5). In seven cases the larvae were allowed to develop from egg to final instar but young larvae do not survive on *P. quadrangularis* and so half-grown larvae were transferred from *P. coerulea* in this case. There were considerable differences in rates of growth and in the dry weight of the larvae as sampled. The results given in Table 5 are arranged in descending weight order. Both the total cyanide content (representing the total cyanoglucoside content) and the amounts of the two component glucosides varied widely. There were no clear correlations between total cyanide and the ratio of linamarin to lotaustralin or between either of these parameters and the dry weight of larvae.

In another experiment the biogenetic precursors in plant biosynthesis of linamarin and lotaustralin, the amino acids valine and isoleucine resp. (Conn, 1979) have been fed together with the normal food plants. This was done with *H. melpomone* larvae fed upon two *Passiflora* species, *P. edulis* and *P. serratadigitata* but with indefinite results. For larvae raised on *P. edulis* the ratio of linamarin varied as would be expected. However, no difference was found in larvae reared upon *P. serratadigitata*. In the latter case the relative amount of lotaustralin was very low and survival of larvae was very poor even though larger larvae were used. This was surprising since *P. serratadigitata* is a normal foodplant of *H. melpomone*, whereas *P. edulis* is not.

Table 5. The quantity of cyanide and proportions of linamarin and lotaustralin found in *H. melpomone* larvae reared on different species of *Passiflora*

Foodplant	Dry matter (mg) Total	Dry matter (mg) per individual	μmol CN^- per gdm	μmol CN^- per individual	Ratio linamarin: lotaustralin
P. antiaquiensis	1130	94.2	37.8	3.57	79:21
P. coerulea	960	80.0	29.0	2.32	84:16
P. edulis	850	70.8	23.7	1.68	87:13
P. laurifolia	847	70.6	27.8	1.96	84:16
P. allardae	818	68.1	28.8	1.96	77:23
P. serratadigitata	806	67.1	15.5	1.04	77:23
P. coccinea	716	59.6	20.1	1.20	77:23
P. quadrangularis	499	41.5	30.8	1.28	84:16

Each sample analysed was of 12 larvae reared from egg to final instar except for *P. quadrangularis*; those larvae were started when half grown.

Table 6. The influence of either 0.1 M valine or 0.1 M isoleucine in 10% sucrose solution fed to imagines of *H. melpomone* on the content of linamarin and lotaustralin compared to the controls (sucrose solution only)

Sample	Number analysed	Food	Dry matter (mg)	μmol CN^- per gdm	μmol CN^- per individual	Ratio linamarin: lotaustralin
Females	9	Valine	463	114	5.8	96:4
	9	Control	534	61	3.6	76:24
	7	Isoleucine	405	63	3.7	31:69
	8	Control	485	51	3.1	85:15
Males	9	Valine	369	127	5.2	92:8
	9	Control	351	75	2.9	84:16
	12	Isoleucine	552	71	3.3	41:59
	9	Control	440	70	3.4	85:15

However, administration of valine and isoleucine to imagines in a sucrose solution via the proboscis indicates a clear positive effect of valine on the content of linamarin and isoleucine on the content of lotaustralin in the glucoside mixture (Table 6). Furthermore, the total cyanoglucoside content is indicated to be higher in treated imagines than in untreated. Again the total content is higher in female than male on the individual basis except untreated males in the isoleucine series. The change in the glucoside proportion is larger in females than in males after treatment with either amino acid.

Biogenetic precursors of linamarin and lotaustralin

Valine and isoleucine were shown to be precursors for cyanoglucoside biosynthesis by administration of both amino acids uniformly labelled with ^{14}C (Table 7). Recovery of activity in the methanolic extract was highest in those larvae that were taken 24 hr after dosage as were the specific activities of the two glucosides, although total cyanide (representing total cyanoglucosides) was at its lowest at that time. Cyanide decreased following pupation but was elevated in female imagines. Also the linamarin to lotaustralin ratio increased with time and so in both these respects the previous results were confirmed. A noticeable aspect of these results is that the incorporation of activity was consistently higher for linamarin than for lotaustralin. Incorporation rates were minimal for the pupal samples and higher in imagines, particularly in females. In the case of linamarin incorporation rate increased to a greater extent than did total cyanide in female imagines with the result that specific activity was also increased. As no further application of ^{14}C precursor occurred the increase can be explained only by using endogenous [^{14}C]valine for *de novo* synthesis, e.g. from proteins.

Imagines were also able to synthesize the cyanoglucosides since active linamarin and lotaustralin were found in imagines after they had been fed ^{14}C-labelled valine and isoleucine via the proboscis (Table 8). Direct comparison between the sexes is not possible since the males were all freshly emerged specimens while females were taken immediately after ovipositing, thus ensuring that all specimens were mature. In both, lotaustralin had a higher specific activity than linamarin. This reflects in part the smaller quantity of lotaustralin compared with linamarin but in the case of the females the incorporation rate for isoleucine is higher than for valine. In this latter respect the results for females differ both from those obtained for males and also for larvae. It had been noted previously (Table 2) that eggs contained the largest proportion of lotaustralin of any stage of the life cycle and so this result may well have arisen because egg formation was taking place.

Apart from that particular result incorporation rates were generally lower than those that had been obtained after dosage of larvae as were the specific activities of linamarin but this is due in part to the greater amounts of linamarin in the imagines compared with larvae.

When labelled valine and isoleucine were administered separately to larvae the results given in Table 9 were obtained. The amount and concentration of cyanide (representing total cyanoglucosides) was lower in larvae that had been injected than in those that had been fed and the ratio of linamarin to lotaustralin was higher. This affects comparisons between results for larvae that had been dosed in different ways. Despite containing greater amounts of both glucosides, the larvae that had been fed with labelled amino acids had as high or higher specific activities in the glucosides and this is reflected in higher incorporation rates, compared with those that had been injected. Comparing larvae that had been fed with pupae, pupae contained more total cyanoglucosides and more linamarin than larvae but less lotaustralin. Specific activities and incorporation rates were lower in pupae, particularly for those dosed with isoleucine.

In every case the activity was located primarily in the glucoside in which it would be expected to occur regarding the biosynthetic precursors for linamarin and lotaustralin which had been recognized in plant biosynthesis for both glucosides (Conn, 1979). This indicates that there can be little if any transfer of activity to the alternate glucoside and that the metabolic processes occurring in the digestive tract or during pupation do not influence the result. This is supported by the results obtained from feeding experiments with ^{13}C-labelled valine and isoleucine (Wray *et al.*, in preparation) which clearly indicate a total incorporation of both amino acids except the car-

Table 7. Incorporation of [^{14}C]valine and [^{14}C]isoleucine into *H. melpomone larvae*

Samples		Radioactivity: Petrol extract (%)	Radioactivity: MeOH extract (%)	Radioactivity: Residue (H^+) (%)	CN^- μmol/individual	Glucosides: Linamarin (%)	Linamarin μmol/individual	Lotaustralin (%)	Lotaustralin μmol/individual	Specific activity (dpm × 10^4): Linamarin	Specific activity (dpm × 10^4): Lotaustralin	Incorporation rate (%): Linamarin	Incorporation rate (%): Lotaustralin
Larvae	24 hr after injection	3	12.5	52	0.91	80	0.728	20	0.182	4.64	6.80	6.14	2.25
Larvae	48 hr after injection	3.8	5.9	64	2.01	83	1.668	17	0.342	2.02	1.36	6.13	0.85
Larvae	72 hr after injection	3.1	4.7	28	1.91	85	1.623	15	0.286	1.40	1.36	4.13	0.71
Pupae	2–3 days after pupation	2.7	6.6	39	1.30	96	1.248	4	0.052	1.40	1.70	3.18	0.16
Imagines ♂	Immediately after emerged	2.4	5.0	42	1.38	97	1.339	3	0.041	1.50	1.30	3.65	0.10
Imagines ♀	Immediately after emerged	2.8	5.3	45	2.10	95	1.995	5	0.105	2.07	1.20	7.51	0.23

Each larva was dosed with 0.25 μCi valine and 0.25 μCi isoleucine by injection into the haemocoele.

Table 8. Incorporation of [^{14}C]valine and [^{14}C]isoleucine into *H. melpomone* imagines

Sample	D.M. (mg)	μmol CN^- per gdm	μmol CN^- per individual	Linamarin (%)	Linamarin μmol/individual	Lotaustralin (%)	Lotaustralin μmol/individual	Specific activity (dpm × 10^4): Linamarin	Specific activity (dpm × 10^4): Lotaustralin	Incorporation rate (%): Linamarin	Incorporation rate (%): Lotaustralin
Imagines mature ♀	426	70.0	2.98	85	2.5	15	0.45	0.6	6.1	3.5	6.0
Imagines fresh ♂	431	70.1	3.02	92	2.8	8	0.24	0.7	3.0	3.9	1.5

A total of 10 female and 10 male imagines were used. The former received 2.08 μCi (~ 4.58 × 10^6 dpm) and the latter 2.28 μCi (~ 5.0 × 10^6 dpm) of both valine and isoleucine.

Table 9. Incorporation of radioactivity into cyanoglucosides in *H. melpomone* larvae given either [^{14}C]valine or [^{14}C]isoleucine both by injection and by feeding

Sample	D.M. (mg)	μmol CN^- per gdm	μmol CN^- per individual	Linamarin (%)	Linamarin Total μmol	Lotaustralin (%)	Lotaustralin Total μmol	Specific activity (dpm × 10^4) Linamarin	Specific activity (dpm × 10^4) Lotaustralin	Incorporation rate (%) Linamarin	Incorporation rate (%) Lotaustralin
Larvae valine (injection)	745	20.5	1.53	94	14.4	6	0.9	3.2	0.01	4.2	0.0001
Larvae isoleucine (injection)	650	24.0	1.56	96	15.0	4	0.62	0.0	2.9	—	0.16
Larvae valine (feeding)	761	40.2	3.06	82	25.1	18	5.5	3.2	0.1	7.3	0.005
Larvae isoleucine (feeding)	625	48.5	3.03	79	24.0	21	6.4	0.02	6.5	0.04	3.8
Pupae valine (feeding)	835	42.6	3.56	93	33.1	7	2.5	2.2	0.015	6.6	0.003
Pupae isoleucine (feeding)	853	43.3	3.69	95	35.0	5	1.8	0.01	2.5	0.03	0.4

Each sample represents 10 specimens, each having been dosed with 0.5 μCi activity. Larvae were taken for analysis 24 hr after dosage and pupae 4 days after pupation, pupation having occurred 3–5 days after dosage.

boxyl group without disconnection of the residual carbon-bonds.

DISCUSSION

Two recent reviews (Brown, 1981; Turner, 1981) show how widely heliconian butterflies have been studied but, in so doing, emphasize that there is a dearth of information on their specialized biochemistry. This is in contrast to the high level of agreement that has been reached on their taxonomic relationships, based upon morphological evidence. Ehrlich (1958) considered that *Heliconiini* did not differ sufficiently from other genera in the *Nymphalinae* to warrant elevation to subfamily rank but that *Acraeinae* did. This placed *Heliconiini* closer to the Paleotropical genera *Cethosia* and possibly also *Vindula*, a point noted by Brown (1981). Of the 10 heliconian genera considered by Brown, Turner (1981) combined three with *Heliconius*, assigning to them subgenera status. *Podotricha* was not mentioned, presumably because the early stages of this genus are not known, but *Philaethria* received particular mention as possibly being closer to the *Cethosia* group of tropical Asia. It has thus been possible to analyse specimens of all heliconian genera, together with their closest relatives (Table 1). In support of the morphological evidence for relationship can now be added the observation that all heliconians and also *Cethosia* and *Acraea* contain the same two cyanoglucosides. Larvae of all the genera examined are reported to feed upon *Passifloraceae* but the same foodplants can also be used by other lepidoptera that are obviously not cyanogenic such as *Parthenos sylvia* and *Vindula arsinoe*.

The presence of very low amounts of linamarin has been reported for only a few species of *Passiflora* (Fung *et al.*, 1981; Fisher *et al.*, 1982). It seems clear that the major cyanoglucosides in this particular plant family are those that contain a cyclopentenyl moiety and so the presence of linamarin and lotaustralin in the insects can only have arisen from *de novo* synthesis. It would be expected that all of the cyanogenic genera possess the capacity to synthesize both glucosides as larvae but this has as yet only been demonstrated conclusively in *Heliconius*. Indeed, it has not yet been possible to test early stages of the other genera for the presence of cyanoglucosides with the exception of pupae of *A. violae* and they proved to be positive. *Heliconius* imagines can also synthesize the glucosides and this could be related in a significant manner to their comparatively long life span that necessitates extensive and specialized adult feeding (Gilbert, 1972).

By similar experimental procedures involving isotopes it has been found that larvae of *Zygaeninae*, the only other family of cyanogenic lepidoptera known (Davis & Nahrstedt, 1982), can also synthesize the same glucosides from valine and isoleucine (Davis & Nahrstedt, unpublished). Thus cyanoglucosides differ from many other compounds of a toxic nature that occur in lepidoptera and that are identical or similar to secondary metabolites of plants (for a recent review see Nahrstedt, 1982) in that they can be synthesized by the insects themselves and do not depend upon sequestration during larval feeding. This specialized aspect of insect biochemistry raises the question of

how much weight should be given to chemical characteristics in taxonomy and whether cyanogenesis denotes a clear dividing line within the *Nymphalinae*. Before this point can be pressed it will be necessary to test for the presence of cyanoglucosides in other genera.

Investigations with plants have revealed that linamarin and lotaustralin occur together because a single enzyme system achieves the synthesis of both glucosides (Hahlbrook & Conn, 1971). The proportions may differ according to the availability of and specificity for the alternative substrates, valine and isoleucine. Since these amino acids have now been shown to be the substrates for cyanoglucoside biosynthesis in *Heliconius* the same considerations may apply. No attempt has yet been made to isolate and characterize the biosynthetic enzyme system and so there is no direct information on substrate specificity. Indirectly it may be noted that in all experiments in which isotopically labelled amino acids were administered to larvae, the incorporation of isotope was always higher for valine (Table 7). This indicates a higher affinity for valine and could be the explanation for the observation that at all times more linamarin is present than lotaustralin. Substrate availability may also affect this and in the one test in which a difference was found, with larvae fed on *P. edulis* loaded with either valine or isoleucine, the difference was in the expected direction. However, additional plant feeding as an uncontrolled food may disturb the results; providing imagines, which accept an artificial diet (sucrose/amino acid solution), with either valine or isoleucine, clearly shows that at least in the adult stage availability of substrates influences the linamarin to lotaustralin ratio as well as the total glucoside content (Table 6).

With imagines the ^{14}C incorporation results were similar. Whilst both male and female are able to synthesize the cyanoglucosides it was surprising that the incorporation rate for isoleucine was higher than the incorporation rate for valine in the mature females (Table 8). Their whole bodies still contained a lower level of lotaustralin than linamarin, consistent with the previous result (Table 2) but a considerably higher proportion of lotaustralin than in female pupae. The freshly emerged males had similar proportions of glucosides to those found in male pupae. The previous result for male imagines had been obtained with older specimens and there was a trend towards a higher proportion of lotaustralin in those. It is possible, therefore, that the proportion of lotaustralin increases with age in imagines of both sexes. Nutrition also may be involved in these changes as indicated above. However, the relatively high incorportation rate of isoleucine in the females is consistent with the much higher proportion of lotaustralin found in eggs (Table 2).

It is quite clear that substantial changes occur at or during pupation. Results from the three experiments in which pupae were analysed all show a decrease in the proportion of lotaustralin, resulting from a decrease in the amount of lotaustralin and an increase in the amount of linamarin. Thus, both synthesis and degradation of glucosides must occur.

As in synthesis, it would be expected that a common enzyme system will be responsible for catabolism of both glucosides. Thus a complex system must exist in which the final proportions of the glucosides depend upon the specificities of both anabolic and catabolic enzyme systems, modulated by substrate availabilities. Again, there is no direct information on the specificity and activity of β-glucosidase in these insects, required for the initial stage of catabolism, but indirect indications are available. In both experiments in which amino acids were given to larvae by injection the level of glucosides found after 24 hr was low. It appears that a loss of cyanide occurred and the apparent extent of the loss was greater than could be accounted for by loss of haemolymph. Also the loss of lotaustralin appeared to be greater than that of linamarin. In the comparison between larvae fed labelled amino acids and injected larvae the quantities of glucoside in the injected larvae were 40 and 80–90% less for linamarin and lotaustralin respectively. Assuming that β-glucosidase is spatially separated from the glucosides in a normal larva it is possible that disruption of tissue caused by the injection procedure results in access of the substrate to the enzyme, or that this is achieved by a defence mechanism. Since lotaustralin is always present in lesser quantities than linamarin, even in haemolymph (Table 4), it would appear that it is the most rapidly degraded of the two glucosides.

These investigations have added to our knowledge of the distribution of cyanoglucosides in butterflies and demonstrated unequivocally that valine and isoleucine are precursors for linamarin and lotaustralin respectively. At the same time additional problems have been raised. Nutritional effects need to be investigated further for both larval and adult stages. There is a need for additional information on age related trends. Most importantly it seems clear that it will not be possible to interpret certain observations unless direct information can be obtained on the enzyme systems responsible for both the synthesis and degradation of the glucosides, information on both their location and their characteristics. Such information would also help towards an understanding of the biological role of cyanoglucosides in these insects.

Acknowledgements—We thank Dr J. R. G. Turner and J. L. S. Stone for providing the foundation stock for the butterfly cultures. The maintenance of the cultures and conduct of experiments owes much to the enthusiastic assistance of A. E. Fermor and C. Ladley. Sexing of pupae was undertaken by Dr M. J. W. Copland. Some samples were supplied by the British Museum via Dr M. Boppré (Univ. of Regensburg) and specimens of *H. charitonia* by Prof. D. Schneider (Max-Planck Institut, Seewiesen) to both of which we give our thanks. We also acknowledge the gift of β-glucosidase preparations from Dr R. Lieberei and D. Selmar (Univ. of Braunschweig).

REFERENCES

BOYDEN T. C. (1976) Butterfly palatability and mimicry: experiments with Ameiva lizards. *Evolution* **30**, 73–81.

BROWER L. P., BROWER J. V. Z. & COLLINS C. T. (1963) Experimental studies of mimicry—7. Relative palatability and Mullerian mimicry among neo-tropical butterflies of the subfamily *Heliconiinae*. *Zoologica NY* **48**, 65–84.

BROWN K. S. JR (1981) The biology of *Heliconius* and related genera. *A. Rev. Ent.* **26**, 427–456.

CONN E. E. (1979) Biosynthesis of cyanogenic glycosides. *Naturwissenschaften* **66**, 28–34.

CONN E. E. (1980) Cyanogenic Glycosides. *Encycl. Plant Physiol.* N.S. Vol. **8,** 461 ff.

CORBET A. S. & PENDLEBURY H. M. (1978) *The Butterflies of the Malay Peninsular* (3rd edn rev., ELIOT. J. N.) Malayan Nature Society, Kuala Lumpur.

DAVIS R. H. & NAHRSTEDT A. (1979) Linamarin and lotaustralin as the source of cyanide in *Zygaena filipendulae* L. (Lepidoptera). *Comp. Biochem. Physiol.* **64B,** 395–397.

DAVIS R. H. & NAHRSTEDT A. (1982) Occurrence and variation of the cyanogenic glucosides linamarin and lotaustralin in species of the Zygaenidae. *Comp. Biochem. Physiol.* **71B,** 329–332.

EHRLICH P. R. (1958) The comparative morphology, phylogeny and higher classification of the butterflies (Lepidoptera: Papilionoidae). *Univ. Kans. Sci. Bull.* **39,** 305–370.

FISCHER F. C., FUNG S. Y. & LANKHORST P. P. (1982) Cyanogenesis in Passifloraceae. Cyanogenic compounds from Passiflora capsularis, P. warmingii and P. perfoliata. *Planta med.* **45,** 42–45.

FUNG S. Y., KHOE K. & FISCHER F. C. (1981) Cyanogenesis in Passiflora spp. *Planta med.* **42,** 122.

GILBERT L. E. (1972) Pollen feeding and reproductive biology of *Heliconius* butterflies. *Proc. natn Acad. Sci. USA* **69,** 1403–1407.

HAHLBROOK K. & CONN E. E. (1971) Evidence for the formation of linamarin and lotaustralin in flax seedlings by the same glucosyltransferase. *Phytochemistry* **10,** 1019–1023.

MARSH N. & ROTHSCHILD M. (1974) Aposematic and cryptic lepidoptera tested on the mouse. *J. Zool. Lond.* **174,** 89–122.

NAHRSTEDT A. (1977) Ersatz karzinogener und allergener Amine in der Cyanidbestimmung nach Aldridge. *Dtsch. Apoth. Ztg.* **117,** 1357.

NAHRSTEDT A. (1982) Strukturelle Beziehungen zwischen pflanzlichen und tierischen Sekundärstoffen. *Planta med.* **44,** 2–14.

NAHRSTEDT A. & DAVIS R. H. (1981a) The occurrence of the cyanoglucosides, linamarin and lotaustralin in *Acraea* and *Heliconius* butterflies. *Comp. Biochem. Physiol.* **68B,** 575–577.

NAHRSTEDT A. & DAVIS R. H. (1981b) Cyanogenic glycosides in butterflies: detection and synthesis of linamarin and lotaustralin in *Heliconiinae*. *Planta med.* **42,** 124–125.

TANTISEWIE B., RUIJGROK H. W. L. & HEGNAUER R. (1969) Die Verbreitung der Blausäure bei den Kormophyten; 5. *Mittlg. Pharm. Weekbl.* **104,** 1341.

TURNER J. R. G. (1981) Adaptation and evolution in *Heliconius*: a defence of neo-Darwinism. *A. Rev. Ecol. Syst.* **12,** 99–121.