Phytochemistry, 1965, Vol. 4, pp. 127 to 131. Pergamon Press Ltd. Printed in England

THE DISTRIBUTION OF THE CYANOGLUCOSIDES LINAMARIN AND LOTAUSTRALIN IN HIGHER PLANTS

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(*Received* 27 *May* 1964)

Abstract—In a survey of plants which have previously been reported to contain either linamarin or lotaustralin, it was found that in general both cyanoglucosides were present. Seeds of *Hevea brasiliensis* were exceptional in that only linamarin could be detected. The two cyanoglucosides were found to be present in a number of additional species drawn from genera containing species already known to contain either linamarin or lotaustralin. Implications of the similar distribution of the two cyanoglucosides are discussed with respect to their biosynthesis.

INTRODUCTION

THE cyanogenic glucosides linamarin, 2-hydroxy-isobutyronitrile-*O*-β-D-glucose, and lotaustralin, 2-hydroxy-2-methyl butyronitrile-*O*-β-D-glucoside, have been reported to occur together in *Trifolium repens* L.[1] and *Lotus australis* Andr.[2] Linamarin has been isolated from several additional plant species in which lotaustralin is not known to occur,[3–5] while lotaustralin—but not linamarin—has been isolated from *L. arabicus* L.[6]

In the course of studies of the biosynthesis of cyanoglucosides,[7] it was observed that linamarin and lotaustralin occurred in approximately equivalent amounts in the tissues of linen flax (*Linum usitatissimum* L.), where previously only linamarin had been reported.[8,9] In both flax[7] and *Trifolium repens*,[10] the synthesis of linamarin and lotaustralin was shown to be closely associated with the metabolism of valine and isoleucine respectively. It therefore seemed likely that lotaustralin has a similar distribution in higher plants to linamarin. A survey has now been carried out and the results are presented in this paper.

RESULTS

Extracts of the plant tissues were prepared and the total cyanoglucoside content was measured by treatment of suitable aliquots with linamarase and determination of the HCN released.

Suitable aliquots were used for paper chromatography in a solvent system which resolves linamarin and lotaustralin. The cyanoglucosides were located by treatment of the papers

[1] J. MELVILLE and B. W. DOAK, *New Zealand J. Sci. Technol.* **22**, 67 (1940).
[2] H. FINNEMORE and J. M. COOPER, *J. Soc. Chem. Ind.* (*London*) **57**, 162 (1938).
[3] W. KARRER, *Konstitution und Vorkommen der organischen Pflanzenstoffe*, Birkhauser Verlag, Basle (1958).
[4] G. DILLEMANN, *Encyclopedia of Plant Physiology*, Vol. 8, p. 1050, Springer Verlag, Berlin (1958).
[5] R. HEGNAUER, *Pharm. Weekblad* **94**, 248 (1959).
[6] T. A. HENRY, *J. Soc. Chem. Ind.* (*London*) **57**, 248 (1938).
[7] G. W. BUTLER and E. E. CONN, *J. Biol. Chem.* **239**, 1674 (1964).
[8] A. JORISSEN and E. HAIRS, *Bull. Acad. Roy. Sci. Belg.* **14**, 923 (1887).
[9] A. JORISSEN and E. HAIRS, *Bull. Classe Sci., Acad. roy. Belg.* **21**, 529 (1891).
[10] G. W. BUTLER and B. G. BUTLER, *Nature* **187**, 780 (1960).

TABLE 1. TOTAL CYANOGLUCOSIDE CONTENT AND RELATIVE AMOUNTS OF LINAMARIN AND LOTAUSTRALIN IN TISSUES OF VARIOUS PLANT SPECIES

Species	Variety or source	Tissue analysed	Cyanoglucoside content (μg HCN released/g fresh weight)	Relative proportions		Cyanoglucoside previously reported
				Linamarin (%)	Lotaustralin (%)	
Linum usitatissimum L.	"Redwood", Calif., U.S.A.	Seedling tops	910	55	45	Linamarin[8,9]
Linum grandiflorum Desf.	Thompson & Morgan (Ipswich) England	Seedling tops	609	62	38	—
Linum perenne L.	Thompson & Morgan (Ipswich) England	Seedling tops	256	63	37	—
Linum narbonense L.	Thompson & Morgan (Ipswich) England	Seedling tops	517	41	59	—
Phaseolus lunatus L.	Wild, large seed, Jamaica	Seed	111	92	8	Linamarin[11]
Phaseolus lunatus L.	Wild, small seed, Jamaica	Seed	89	96	4	—
Trifolium repens L.	Twenty collections	Young leaves	3·2–352	26–50	50–74	Both present[1]
Lotus arabicus L.	Morocco	Seedling tops	370	70	30	Lotaustralin[6]
Lotus arenarius Brot.	Morocco	Seedling tops	879	99	1	—
Lotus corniculatus L.	"Cascade", Washington, U.S.A.	Seedling tops	105	57	43	—
Lotus corniculatus L.	"Los Banos Trefoil", Calif., U.S.A.	Seedling tops	24	11	89	—
Lotus corniculatus L.	Morocco	Seedling tops	56	54	46	—
Lotus creticus L.	Morocco	Seedling tops	151	84	16	—
Lotus edulis L.	Morocco	Seedling tops	178	79	21	—
Lotus maroccanus Ball.	Morocco	Seedling tops	292	58	42	—
Lotus maroccanus Ball.	Morocco, C.S.I.R.O. No. 22801	Seedling tops	79	74	26	—
Lotus parviflorus Desf.	Botany Div., D.S.I.R., N.Z.	Seedling tops	101	21	79	—
Lotus tenuis Waldst. et Kit. ex. Willd.	Christchurch, N.Z.	Seedling tops	88	6	94	—
Manihot carthaginensis Muell. Arg.	Christchurch Bot. Garden, N.Z.	Roots	—	96	4	—
Hevea brazilienvis Muell. Arg.	Barcelona Bot. Garden, Spain	Seed	91	100	0	Linamarin[12]
Dimorphotheca ecklonis DC	Harrison's Nurseries, Palmerston North, N.Z.	Young leaves	1580	100	trace	Linamarin[13]
Dimorphotheca barberae Harv.	Harrison's Nurseries, Palmerston North, N.Z.	Young leaves	1210	100	trace	—
Osteospermum jucundum Norlindh.	Harrison's Nurseries, Palmerston North, N.Z.	Young leaves	1045	100	trace	—

[11] W. R. DUNSTAN and T. A. HENRY, *Proc. Roy. Soc.* **72**, 285 (1903).
[12] K. GORTER, *Rec. Trav. chim.* **31**, 264 (1912).
[13] L. ROSENTHALER, *Schweiz. Apoth.-Ztg.* **60**, 234 (1922).

with linamarase prepared from linseed meal, followed by detection of both the liberated HCN and glucose. The amounts of linamarin and lotaustralin present were measured by determining the amount of glucose liberated in each case.

Table 1 shows the results of the survey. It will be seen that the relative contents of the two cyanoglucosides present varied widely between species and varieties.

The four *Linum* species examined contained considerable amounts of both linamarin and lotaustralin. With *Trifolium repens*, where twenty collections were tested, the total cyanoglucoside content varied 100-fold but the relative proportions of linamarin and lotaustralin did not vary greatly. Linamarin and lotaustralin were both present in all of the cyanogenic *Lotus* species and varieties tested, but there was wide variation in the relative proportions of the two cyanoglucosides. With *Phaseolus lunatus*, *Manihot carthaginensis*, *Dimorphotheca ecklonis* and *D. barberiae* and the closely related *Osteospermum jucundum*, linamarin was present to a much greater extent than lotaustralin. Linamarin has previously been reported from roots of *M. utilissima* Pohl and *M. palmata* Muell. Arg.[14] and from leaves of *D. spectabilis* Schl't. and *D. zeyheri* Sond.,[15] *D. cuneata* Less.[16] and *D. fructicosa* DC.[17] No lotaustralin was detected in seed of *Hevea brasiliensis*.

In the case of *Osteospermum jucundum* where only a trace of lotaustralin could be detected, further evidence for the presence of linamarin and lotaustralin was obtained by means of ^{14}C-labelling experiments. Uniformly labelled L-valine-^{14}C and L-isoleucine-^{14}C were administered to freshly excised young leaves, which were allowed to metabolize for 20 hr. The material was then extracted, two-dimensional chromatograms prepared and radioautographs made. Strongly labelled radioactive areas were observed in positions corresponding to linamarin in the case of L-valine-^{14}C administration and lotaustralin in the case of L-isoleucine-^{14}C administration. Also, the general labelling patterns observed on the radioautographs were very similar to those previously observed in experiments with *Linum usitatissimum*.[7] The radioactive areas corresponding to the cyanoglucosides were eluted, the radioactivity was determined and the cyanoglucosides decomposed by treatment with linamarase. Upon determination of the residual radioactivity it was found that observed counts per minute were reduced by this treatment from 3128 to 20 for linamarin and 2182 to 15 for lotaustralin (corrected for background). This is consistent with the formation of volatile products (acetone or methyl ethyl ketone and HCN) from the ^{14}C-labelled aglycone moieties of the two cyanoglucosides. Furthermore, the presence of glucose in the residues was established by paper chromatography.

DISCUSSION

Isolation and characterization of linamarin and lotaustralin from the various plant species was not attempted in this survey. The establishment of the presence of two cyanoglucosides is unequivocal, since it rests on the identification on paper chromatograms of both HCN and glucose liberated by the action of linamarase. The identification of these two glucosides as linamarin and lotaustralin rests on paper chromatography in five solvent systems together with their ease of hydrolysis by linamarase. In addition, the ready incorporation of carbon-14 from L-valine-^{14}C and L-isoleucine-^{14}C into linamarin and lotaustralin

[14] W. R. Dunstan and T. A. Henry, *Proc. Roy. Soc.* **78**, 145 (1906).
[15] C. Rimington, *18th Rep. Director Vet. Serv. Animal Ind., Onderstepoort*, p. 955 (1932).
[16] J. S. C. Marais and C. Rimington, *Onderstepoort J. Vet. Sci. Animal Ind.* **3**, 111 (1934).
[17] C. Rimington and D. G. Steyn, *Onderstepoort J. Vet. Sci. Animal Ind.* **5**, 79 (1935).

respectively confirms the identification of these two cyanoglucosides for *Linum usitatissimum*,[7] *Trifolium repens*[10] and *Osteospermum jucundum* (this paper).

While it appears to be true that the distribution of lotaustralin is closely similar to that of linamarin, the latter cyanoglucoside is preferentially synthesized in species of several genera. It is of interest that in one of the latter species. (*O. jucundum*). there was extensive incorporation of carbon-14 from L-isoleucine-^{14}C into the aglycone moiety of the trace of lotaustralin present.

Closely similar or identical biosynthetic pathways involving valine and isoleucine metabolism have been shown to operate in *Trifolium repens*. *Linum usitatissimum* and *Osteospermum jucundum* members of the families Leguminosae, Linaceae and Compositae respectively. It seems reasonable to expect that the same metabolic routes would be present in cyanogenic species of *Phaseolus*, *Lotus*, *Manihot* and *Dimorphotheca*. where both cyanoglucosides have been shown to be present. The position with *Hevea brasiliensis* requires further study. If lotaustralin is indeed absent from this tissue, either the enzymes involved in the biosynthesis have greater substrate specificity in *H. brasiliensis*. or linamarin is here synthesized by a different route.

EXPERIMENTAL

The *Trifolium repens* material used was from a trial carried out by Mr. G. S. Harris, Grasslands Division, D.S.I.R., where 20 collections of *T. repens* originating from the Mediterranean area, Europe, North America and New Zealand were growing in the field on a randomized layout. *Linum* and *Lotus* species were grown from seed in pots for 3–6 weeks under glass and the seedling tops were harvested for analysis. For *T. repens*. *Dimorphotheca* sp. and *Osteospermum jucundum*, young leaves were selected from vigorously growing plants. For *Manihot carthaginensis*, roots were freed of adhering soil particles and extracted as described below. Seed of *Phaseolus lunatus* and *Hevea brasiliensis* were first ground in a Casella seed-mill.

Extractions of 1–2 g fresh weight of plant material were made with 50 vol. boiling 80% (v/v) aq. ethanol for 5 min. The solvent was evaporated *in vacuo* at 40 and the residue extracted with 2 ml 10% (v/v) aq. isopropanol. The extract was clarified by centrifugation if necessary and suitable aliquots were used for paper chromatography.

For routine use the solvent system methyl-ethyl ketone:acetone:water (30:10:0·6 v/v) was employed using the descending technique. For two-dimensional chromatography propanol:water (7:3 v/v) was used as the second solvent. Other useful solvents were butanol:pyridine:water (6:4:3 v/v), isopropanol:acetic acid:water (70:5:25 v/v), butanol:acetic acid:water (120:30:50 v/v). R_f data for these solvent systems have been listed elsewhere.[7]

Linamarin and lotaustralin were detected on chromatograms by spraying the paper lightly with a solution of linamarase (purified approximately 20-fold from linseed meal by the method of Coop).[18] The liberated HCN was then detected on an adjacent paper sprayed with alkaline picrate as described elsewhere[10] or the liberated glucose was determined quantitatively using the adaptation[19] of the detection method using aniline phosphate reagent.[20]

[18] I. E. COOP, *New Zealand J. Sci. Technol.* **22B**, 71 (1940).
[19] C. M. WILSON, *Analyt. Chem.* **31**, 1199 (1959).
[20] B. H. HOWARD. *Biochem. J.* 67, 643 (1957).

For the ^{14}C-labelling experiments, uniformly labelled L-valine-^{14}C and L-isoleucine-^{14}C were purchased from Amersham Radiochemical Centre, England. Five microcuries of each amino-acid (0·2 μmole L-valine, 0·8 μmole L-isoleucine) were administered in 0·2 ml water to 1·5 g fresh weight of young leaves through the freshly excised petioles. Radioautographs of two-dimensional chromatograms of the extracts were made using Kodak No-Screen X-ray film with exposure times of 2 weeks.

Radioactivity determinations of eluted cyanoglucosides were made using a Geiger–Muller detector with mica end-window of density 2 mg/cm^2, after drying the eluates on to planchets using an i.r. lamp. The cyanoglucosides were then decomposed by addition of 0·2 ml linamarase in 0·01 M phosphate buffer, pH 6·0, and incubation overnight in the presence of toluene vapour. The liquid was evaporated off the planchets using an i.r. lamp and residual radioactivity was measured.

Acknowledgements—The author thanks Mrs. J. Foot for technical assistance and Miss M. J. A. Simpson and Mr. G. S. Harris for assistance in obtaining plant material.