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Spontaneous Phloem Bleeding from Cryopunctured Fruits of a Ureide-Producing Legume[1]

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ABSTRACT

The vasculature of the dorsal suture of cowpea (*Vigna unguiculata* [L.] Walp) fruits bled a sugar-rich exudate when punctured with a fine needle previously cooled in liquid N_2. Bleeding continued for many days at rates equivalent to 10% of the estimated current sugar intake of the fruit. A phloem origin for the exudate was suggested from its high levels (0.4–0.8 millimoles per milliliter) of sugar (98% of this as sucrose) and its high K^+ content and high ratio of Mg^{2+} to Ca^{2+}. Fruit cryopuncture sap became labeled with ^{14}C following feeding of [^{14}C]urea to leaves or adjacent walls of the fruit, of $^{14}CO_2$ to the pod gas space, and of [^{14}C] asparagine or [^{14}C]allantoin to leaflets or cut shoots through the xylem. Rates of translocation of ^{14}C-assimilates from a fed leaf to the puncture site on a subtended fruit were 21 to 38 centimeters per hour. Analysis of ^{14}C distribution in phloem sap suggested that [^{14}C]allantoin was metabolized to a greater extent in its passage to the fruit than was [^{14}C] asparagine. Amino acid:ureide:nitrate ratios (nitrogen weight basis) of NO_3-fed, non-nodulated plants were 20:2:78 in root bleeding xylem sap *versus* 90:10:0.1 for fruit phloem sap, suggesting that the shoot utilized NO_3-nitrogen to synthesize amino acids prior to phloem transfer of nitrogen to the fruit. Feeding of $^{15}NO_3$ to roots substantiated this conclusion. The amino acid:ureide ratio (nitrogen weight basis) of root xylem sap of symbiotic plants was 23:77 *versus* 89:11 for corresponding fruit phloem sap indicating intense metabolic transfer of ureide-nitrogen to amino acids by vegetative parts of the plant.

The ability to recover exudates directly from phloem of a plant species by the aphid stylet technique (5, 10, 24) or by cutting of vascular tissues of stems, petioles, or fruits offers an effective experimental approach for examining the contents of translocation streams (18, 30), for use in conjunction with labeling studies of plant transfer processes (18, 22), and, in a wider context, for studying solute interchange between plant parts during growth and development (15, 29).

In studies of fruit nutrition of legumes, such techniques have been applied successfully in *Pisum*, *Lupinus*, *Spartium*, and *Jacksonia* (23). All of these bleed spontaneously from cut fruit vascular tissue, and are characterized by amide transport in xylem and phloem. More recently, fruits of soybean (*Glycine max*), a ureide-producing legume, have been shown to release solutes of supposed phloem origin when cut under an EDTA solution (9, 14), and a technique has also been described for collecting phloem-derived materials from seed coats of the same species (27).

This paper reports on a novel 'cryopuncture' technique in which the vasculature of the dorsal suture of fruits of cowpea (*Vigna unguiculata*) is pierced with a fine needle cooled in liquid N_2, thereby inducing spontaneous release of sugar-rich fluid from the puncture site. The evidence presented indicates that the exudates originate from phloem, and data from a series of labeling studies provide information on likely pathways and processes for phloem transfer of organic solutes to fruits. Comparisons are made of the composition of these cryopuncture exudates and root xylem bleeding sap of non-nodulated NO_3-fed plants and nodulated N_2-dependent plants to provide information on the major transformations among nitrogenous solutes accompanying N transfer to the fruit under these contrasting N regimes.

MATERIALS AND METHODS

Plants of cowpea (*Vigna unguiculata* [L.] Walp cv Vita 3) were grown in sand culture in a naturally lit glasshouse during February to May in Perth, Western Australia. Some plants (symbiotic, N_2-dependent treatment) were inoculated with the effective *Rhizobium* strain CB756 and received N-free culture solution. Other plants (non-nodulated, NO_3-fed treatment) were not inoculated and received culture solution containing 10 mM NO_3^-; a NO_3^- regime previously shown (3) to suppress nodulation and support a rate of growth and N accumulation comparable to that of companion N_2-dependent plants. All fruits developing at the lowest reproductive node (node 4 [see 21]) were tagged at anthesis and experiments conducted when these fruits had reached an age of 12 to 15 d.

Collection of Phloem Sap by Cryopuncture of Fruits. The standard procedure selected for obtaining phloem exudates was to puncture the vascular strands of the mid-dorsal suture of a fruit with the tip of a fine (0.5-mm) needle previously cooled in liquid N_2, retaining the needle tip in the fruit for a period of 5 s to allow chilling of tissues in the immediate vicinity of the puncture. This treatment induced bleeding usually within 30 min of puncture and by placing microcapillaries (1–5 μl capacity) over the wound, the exudate was readily collected. Collection periods ranged from 30 min to several days depending on the nature of the experiment. Time courses of bleeding (labeled or unlabeled sap) from single sites were followed by regularly changing microcapillaries trapping the exuded sap. Cryopuncturing of fruits was attempted day and night on fruits aged 6 to 18 d after anthesis. Sap collected over several hours of bleeding from 30 or more similar fruits was combined to provide samples of 0.5 to 1.5 ml, enabling a variety of analyses to be conducted on a single sap sample.

Labeling Studies of Origin of Fruit Phloem Sap Solutes. The mode of application and nature of labeled substrates for the various feeding experiments are described pictorially in Figure 1. Details of substrate concentration and isotope content as well as time courses of collection of fruit phloem exudate are provided in the description of individual experiments. [^{14}C]Urea was ap-

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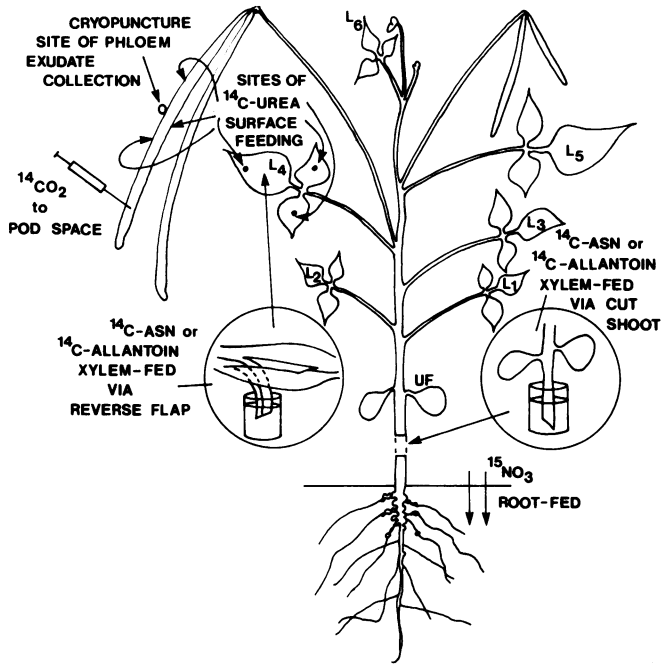

FIG. 1. Diagrammatic representation of sites and modes of application of ^{14}C- or ^{15}N-labeled substrates to intact plants or cut shoots of cowpea. In all cases, phloem exudate was collected from fruits at sites of cryopuncture. UF denotes unifoliate leaflets and L_1 to L_6 leaflets at each of the trifolitate leaves. Details of individual labeling experiments may be found in the text.

plied as a 5-μl drop to a leaf or fruit surface previously wetted with dilute aqueous surfactant (Triton X-100; 0.01%, v/v), $^{15}NO_3$ was added to the rooting medium of intact plants (marked 'root feeding,] Fig. 1), $^{14}CO_2$ injected into the gas space of the fruit (marked 'POD space,' Fig. 1) and [U-^{14}C]Asn or [2-^{14}C]allantoin dissolved in dilute, unlabeled xylem sap, and applied through the transpiration stream to the cut base of a shoot (marked 'cut shoot,' Fig. 1), or through a flap cut in the midvein of a leaflet (marked 'reverse flap' feeding, Fig. 1). All feeding experiments were conducted in daytime under conditions of full illumination using fruits of 12 to 15 d age. All but the $^{15}NO_3$-feeding study utilized nodulated N_2-dependent plants. [2-^{14}C]Allantoin was prepared from [2-^{14}C]uric acid and purified as described previously (4). Feeding aphids were collected from fruits and extracted as described previously for ^{14}C following supply of [^{14}C]urea to the subtending leaflets (4).

Analyses of Solutes of Sap. Total sugars were determined by a phenol-sulfuric acid procedure (21). Individual sugars were identified and assayed as TMS[2] derivatives (11) by flame ionization-GLC following separation on a 2 × 1850 mm glass column of 2% OV-17 on 85 to 100 mesh Chromosorb G, temperature programmed at 4° C/min over the range 150 to 290°C. Organic acids were separated from neutral and basic compounds in exudate samples (2) and resolved by GLC following derivatization with *N,O*-bis(TMS)acetamide in pyridine, using the same chromatographic system as for sugars but temperature programmed at 6° C/min from 80 to 250° C. Sugars and organic acids were identified by co-chromatography with TMS derivatives of authentic compounds. Ureides (allantoin and allantoic acid) were assayed together as the phenylhydrazone derivative of glyoxylate (4) and NO_3 as NO_2, following reduction with Cd/Cu. Ninhydrin-positive amino compounds were separated and estimated on a Beckman 118C amino acid analyzer, operating in the low temperature, physiological fluids (Li-based buffers) mode. Minerals (K, P, Mg, Ca, Fe, Mn, Cu) were measured as described elsewhere for phloem sap analyses (12, 23). Total N and total C were determined on bulked (0.5 to 1.5 ml) samples of phloem or xylem sap by standard Kjeldahl analysis and a Shöniger combustion technique respectively.

Isotopic Analysis of Labeled Fruit Phloem Sap. Aliquots of sap were analyzed for ^{14}C-ureides and [^{14}C]urea by collecting the $^{14}CO_2$ released from hydrolyzed extracts treated with purified jackbean urease (Sigma) in 1 M Hepes-NaOH buffer (pH 8) (4). Distribution of ^{14}C in the sugar plus organic acid fraction and in individual amides and amino acids was studied by separating these compounds on the amino acid analyzer, monitoring for ^{14}C in the eluate stream by means of a scintillation flow cell. ^{15}N was assayed by optical emission spectroscopy (25) following steam distillation of ammonia from Kjeldahl digests of samples of phloem exudates or eluate fractions from the amino acid analyzer.

RESULTS

Induction of Bleeding and the Exudation Phenomenon. Spontaneous bleeding from vascular tissue of cowpea fruits was discovered accidentally while inspecting pods which had been infected by a fruit end-rot organism (*Rhizopus* spp.). As the fungal infection spread from the distal tip of the fruit, drops of fluid were observed to exude from the dorsal suture of the pod close to the transparent, waterlogged tissue marking the advance of the fungus. The exudate consisted of 25 to 30% (w/v) sugar, almost all as sucrose, suggesting an origin from phloem tissue rendered "leaky" by the infection. In an attempt to induce similar leakage from fruits by artificial means, a variety of treatments was tested including cutting of vascular tissue, application to the pod vasculature of chemical agents likely to induce loss of permeability, and localized application of cold. The only treatment effective in promoting exudation from the fruit was puncturing of its vascular tissue with a fine needle previously cooled by immersion in liquid N_2. This treatment, hereafter referred to as 'cryopuncturing,' caused rapid local loss of permeability at the puncture site, followed, in a proportion of cases, by massive exudation of sugar-rich fluid from the wound. The first drops of exudate usually formed within 0.5 h of the cryopuncture and continued for many hours. In some cases bleeding persisted for a week.

Bleeding following cryopuncture was obtained consistently only from the vascular strands of the dorsal suture of the pod, but also occurred occasionally from the vascular tissue of the top of the fruit stalk close to the point of attachment of the fruit. Despite repeated attempts, the strand of the ventral suture or lateral strands of the pod wall could not be induced to exude sugar-rich fluids, nor could exudates be obtained from stems, petioles, or the major veins of leaflets.

Fruits less than 6 d old failed to respond to cryopuncture of their dorsal suture while from those 6 to 9 d old bleeding could be induced, but only with a low success rate. From 9 d onwards, as seeds started to fill, bleeding occurred with high intensity and long duration from the majority of a sample of fruits and from virtually any location along the dorsal suture. Once starting to yellow and desiccate (18–20 d after anthesis) pods failed to bleed. Bleeding was induced most readily in fruits cryopunctured in the late afternoon, exudation commencing usually at dusk. In the early morning of hot dry days, cryopuncture led to less copious bleeding or even to no bleeding that day, although a proportion of the pods commenced to bleed or accelerate their rate of bleeding the following evening. Similarly, volumes of bleeding fluid obtained from a puncture site over a sequence of several days exudation were often noticeably greater at night than during the day. These observations indicated that the bleeding phenom-

[2] Abbreviation: TMS, trimethylsilyl.

enon was markedly affected by diurnal changes in the water status of tissues close to the puncture site.

The exudation caused by cryopuncturing of fruit vascular tissue of cowpea was clearly distinct from the release of pressure sap which regularly occurs from cut, nonvascular tissues of any part of the shoot of this and other legume species (*e.g. Glycine* [see 14] *Phaseolus* and *Kennedia* spp.). This nonvascular sap is opalescent, contains some mucilage, but virtually no sucrose (total sugars less than 0.3%, w/v). Unlike the cryopuncture phloem exudates, it is produced for only a few minutes, is very bitter to the taste and discolors readily, presumably due to oxidation of phenolic compounds.

Solute Composition of Exudate. All fruit exudate samples showed more than 95% of their soluble sugar content to consist of sucrose, while adjacent pod tissue and sap collected from cut fruit tips immersed in EDTA showed monosaccharides as the dominant class of sugars (see also data for soybean fruits (14)). Sucrose levels ranged from 140 to 280 mg/ml (0.41 to 0.82 mmol/ml) with no marked tendency for concentrations to fall noticeably with time after fruit puncture. For example during a 24-h period of bleeding, a set of 10 fruits (15 d old) produced sap with a sucrose concentration of 212 ± 29 mg/ml ($\bar{x}$ ± SE), in the second 24-h period 229 ± 15 mg/ml, and in the 3rd day 202 ± 21 mg/ml.

Maximum volumes of exudate obtained from a single puncture of 12 to 15-d-old fruits were 150 μl/d, a loss equivalent to 30 to 40 mg sucrose and approximately 10% of the current intake of translocate by the fruit during the day. Bleeding thus created a significant artificial sink within a fruit and, with time, reduced significantly the final size of the seeds close to a puncture site. Protein levels of the exudates were less than 0.5 mg/ml. pH of the exudate was 6.5 to 7.0 and not noticeably different between NO_3-fed and symbiotic plants. A typical analysis of organic acids showed 11.6 mg (111 μmol) malonate, 1.4 mg (12 μmol) succinate, 2.3 mg (17 μmol) malate, 0.3 mg (2 μmol) tartarate, and 0.3 mg (1.4 μmol) citrate/ml exudate. In addition, two unidentified acidic compounds were detected. Mineral analyses showed K at 950 to 1360 μg/ml (24.3 to 34.8 μmol/ml), Mg 400 to 470 μg/ml (16.5 to 19.3 μmol/ml), P at 120 to 235 μg/ml (3.9 to 7.6 μmol/ml), Fe at 80 to 177 μg/ml (1.4 to 3.2 μmol/ml), Ca at 53 to 107 μg/ml (1.3 to 2.7 μmol/ml), Mn at 34 to 68 μg/ml (0.6 to 1.2 μmol/ml), and Cu at 16 to 30 μg/ml (0.2 to 0.5 μmol/ml).

Amino acid and ureide contents of fruit exudate varied between symbiotic and NO_3-fed plants and differed substantially from root bleeding xylem sap of the parent plants (Table I). Nitrate was not detected in fruit phloem exudates of NO_3-fed plants but comprised 78% of N of the root bleeding sap from these plants. The ratio of amino acid-N to ureide-N was 7.9:1 in fruit phloem exudate of symbiotic plants, contrasting with 1:3.5 for root bleeding sap of the same plants. In NO_3-fed plants, this ratio was closely similar for fruit phloem exudate (9:1) and root xylem sap (10:1). Ureides were at lower concentration in fruit phloem exudate than in root xylem sap of symbiotic plants, but more than 20 times more concentrated in phloem than in xylem of NO_3-fed plants. Under both forms of N nutrition, amino acids were considerably more concentrated in phloem exudate than in xylem sap.

Amino acid composition of fruit phloem exudate and root xylem sap of NO_3-fed and symbiotic plants (Table II) indicated that amides (Asn and Gln) were the major solutes, accounting for 57% of total sap ninhydrin-positive N. Asn was present at much higher relative level than Gln in the xylem sap of NO_3-fed plants but at lower level than Gln in the other three sap samples. Of the non-amide amino acids, Arg, Asp, and γ-Abu each comprised greater proportions of sap amino-N of xylem than of phloem, while the reverse held for His, Ser, Thr, Val, Ala, and Phe. Proportions of sap N as Ile, Leu, and Tyr were not noticeably different between xylem and phloem or between NO_3-fed and symbiotic plants, while Lys was relatively more prominent in xylem and phloem of NO_3-fed than of symbiotic plants. Amino acids present in trace amounts (0.1–1.0% sap amino-N) in xylem and phloem of both sets of plants were Glu, Pro, Gly, and the sulfur amino acids, Cys and Met.

Assays of bulk samples (1.0–1.5 ml) of fruit phloem exudate for total C, total N, amino compounds, ureides, and sucrose gave estimates of the proportions of the sap C and N accounted for as these sets of compounds. The data (Table III) for five such comparisons showed that sucrose represented from 79 to 91% of the sap total C, the C of sucrose, ureides, and amino compounds from 83 to 96% (mean 88) of sap C. For N, estimates based on amino compounds plus ureides accounted for from 81 to 122% (mean 98%) of the measured Kjeldahl N, suggesting that little N was present in exudates as other compounds. The comparisons of C:N ratio based on Shöniger:Kjeldahl analysis *versus* a ratio calculated as the sum of C in sucrose, amino compounds, and ureide and the sum of N in amino compounds and ureides suggested that on balance solute analysis underestimated the ratio. This might be expected as a C value for organic acids was not included in the estimates given in Table III.

^{14}C- and ^{15}N-Labeling Studies of the Origin of Fruit Phloem Solutes. Appearance of recently formed photosynthate in the cryopuncture sap of fruits was studied by collecting exudate at half-hourly or hourly intervals from plants whose illuminated subtending leaf had been fed [^{14}C]urea (Fig. 1). Typical time courses of labeling (Fig. 2) showed detectable ^{14}C in exudate within 1 to 1.5 h of application of labeled urea to the leaf and peak labeling at this site within the period 1.25 to 2.25 h. Specific activity of the sap then declined. Over 95% of the ^{14}C of the fruit phloem exudate was recovered as sucrose, indicating that, as in earlier studies (23), [^{14}C]urea had been degraded by the active urease of the leaflets (4) and the resulting $^{14}CO_2$ fixed photosynthetically and loaded onto the phloem as sugar. The total yield of ^{14}C from the fruit cryopuncture exudate over a 6-h period was equivalent to 3 to 9% of the ^{14}C applied to the nurse leaf, *versus*

Table I. *Nitrogenous Solutes of Fruit Cryopuncture (Phloem) Exudate and Root (Xylem) Bleeding Sap of Symbiotically Dependent and NO_3-Fed (Non-nodulated) Cowpea*

Nutrition	Sap Type	Nitrogenous Compounds								
		Amino acids			Ureides			Nitrate		
		μg N/ml	*% sap N*	*μmol/ml*	*μg N/ml*	*% sap N*	*μmol/ml*	*μg N/ml*	*% sap N*	*μmol/ml*
Symbiotic	Fruit phloem exudate[a]	2239	89	112	284	11	5.1	ND[b]	ND	ND
	Root xylem sap[c]	135	23	5.5	472	77	8.4	ND	ND	ND
NO_3-fed	Fruit phloem exudate[a]	3973	90	173	398	10	7.1	4	0.1	0.3×10^{-3}
	Root xylem sap[c]	138	20	5.7	15	2	0.3	211	78	15.1

[a] Bulked sample for first 2 h of bleeding from 30 fruits of age ranging from 10 to 14 d after anthesis.

[b] ND, not detected in assay. Lower limit of detection equivalent to 0.1 μg N/ml.

[c] Bulked sample from the same plants used for phloem exudate collection.

Table II. *Percentage Composition and Absolute Levels of Ninhydrin-Positive Amino Compounds of Fruit Cryopuncture (Phloem) Exudate and Root (Xylem) Bleeding Sap of Symbiotically Dependent and NO_3-Fed (Non-nodulated) Cowpea*

Percentage column shows % total ninhydrin-positive N.

Amino Compound	Fruit Phloem Exudate[a] (Symbiotic)		Fruit Phloem Exudate[a] (NO_3-fed)		Root Xylem Sap[b] (Symbiotic)		Root Xylem Sap[b] (NO_3-fed)	
	%	μmol/ml	%	μmol/ml	%	μmol/ml	%	μmol/ml
Amides								
Glutamine	29.9	9.01	30.7	8.10	37.4	1.78	15.8	1.23
Asparagine	22.5	6.77	26.7	7.03	22.5	1.07	40.9	3.18
Total amide	52.4		57.4		59.9		56.7	
Non-amide amino acids								
Arginine	1.8	0.28	2.7	0.36	17.4	0.41	10.9	0.43
Histidine	9.1	1.84	9.4	1.66	1.7	0.05	4.9	0.25
Aspartic acid	0.4	0.26	0.6	0.31	5.1	0.49	7.5	1.17
Serine	5.9	3.53	4.5	2.40	0.4	0.04	1.0	0.16
Valine	5.3	3.20	4.2	2.19	2.6	0.25	1.8	0.28
Lysine	0.1	0.01	3.8	0.10	0.3	0.02	5.1	0.40
Threonine	4.9	2.97	3.1	1.61	1.1	0.11	1.3	0.21
γ-Aminobutyrate	0.9	0.54	0.1	0.08	4.0	0.38	1.8	0.28
Alanine	3.7	2.22	2.0	1.06	0.4	0.04	0.3	0.05
Isoleucine	3.4	2.04	3.4	1.79	2.3	0.22	2.2	0.34
Leucine	3.4	2.04	3.3	1.75	2.1	0.20	2.2	0.34
Phenylalanine	3.1	1.85	1.9	1.01	0.5	0.05	0.6	0.10
Tyrosine	1.7	1.03	0.9	0.49	0.3	0.03	0.6	0.09
Other minor compounds[c]	3.9	2.35	2.7	2.24	1.9	0.18	3.1	0.45

[a] Bulked sample for first 2 h of bleeding from 20 fruits of age ranging from 12 to 15 d after anthesis.
[b] Bulked sample from the same plants used for phloem exudate collection.
[c] Includes Glu, Pro, Gly, Cys, and Met.

Table III. *Total C and N Concentrations of Phloem Exudates from Cowpea Fruits with Levels of These Elements in Identified Sugars and Nitrogenous Solutes of the Exudate*

	Sap Sample Number[a]				
	1	2	3	4	5
Total C (Shöniger combustion) (mg/ml)	82.7	97.4	91.8	88.0	121.6
C as sucrose (S) (mg/ml)	65.5	81.6	77.1	80.0	99.0
C as amino compounds (AA) + ureides (U) (mg/ml)	3.4	2.0	4.3	2.3	4.9
C of S + AA + U as % of total C[b]	83.3	85.8	88.6	96.2	85.4
Total N (Kjeldahl digestion) (mg/ml)	1.14	0.99	1.90	1.50	2.01
N as AA (mg/ml)	1.10	0.66	1.41	1.50	1.59
N as U (mg/ml)	0.13	0.13	0.22	0.33	0.31
N accounted for as U + AA (%)	107.9	80.8	85.8	122.5	94.6
C:N ratio (Shöniger:Kjeldahl)	72.5	98.4	48.3	58.5	60.5
C:N ratio (S + AA + U:AA + U)	56.0	104.5	49.9	46.0	54.6

[a] All samples of 1.0 to 1.5 ml initial volume representing 24-h collection periods from 30 plants.
[b] Sample size precluded assays of organic acids which in other analyses accounted for 10 to 15% of sap total C.

less than 0.1% in the case of EDTA-prompted exudation from fruit tips of comparably labeled plants over the same period of time. The combined path lengths for assimilates from leaflet through petiole and peduncle to the puncture site on the fruit were from 471 to 571 mm, giving estimated translocation times of 21 to 38 cm/h for the arrival of the peak of the front of labeled assimilates in the fruit from the fed leaf. In similar experiments, aphids were collected from fruits following application of [^{14}C] urea to the subtending leaf (L_4). The time course of ^{14}C recovered in extracts of the feeding aphids indicated a similar translocation time for labeled assimilates to the fruit as was found for sampling by cryopuncture exudate collection.

Labeling of fruit phloem sap following feeding of [^{14}C]Asn or [^{14}C]allantoin to cut shoots through the transpiration stream or to the xylem of a leaf was followed in a series of feedings of plants bearing 12- to 15-d-old fruits. Time courses of transfer to fruit phloem sap of ^{14}C were essentially as with leaf-fed [^{14}C] urea. Sap bulked over a 6-h period after feeding showed distributions of label among different classes of solutes as shown in Table IV. The data indicated effective bulk transfer of unmetabolized Asn from the transpiration stream or leaf to the phloem stream serving the fruits, with greater evidence of metabolism of the amide following feeding to the leaflet than when applied through the cut end of the shoot. Transfer of ^{14}C to the sugar plus organic acid fraction of the phloem and to certan phloem amino acids resulted from breakdown of the [^{14}C]Asn. Label in compounds other than Asn represented 46% of phloem sap ^{14}C following feeding of leaves, *versus* only 16% with shoot feeding

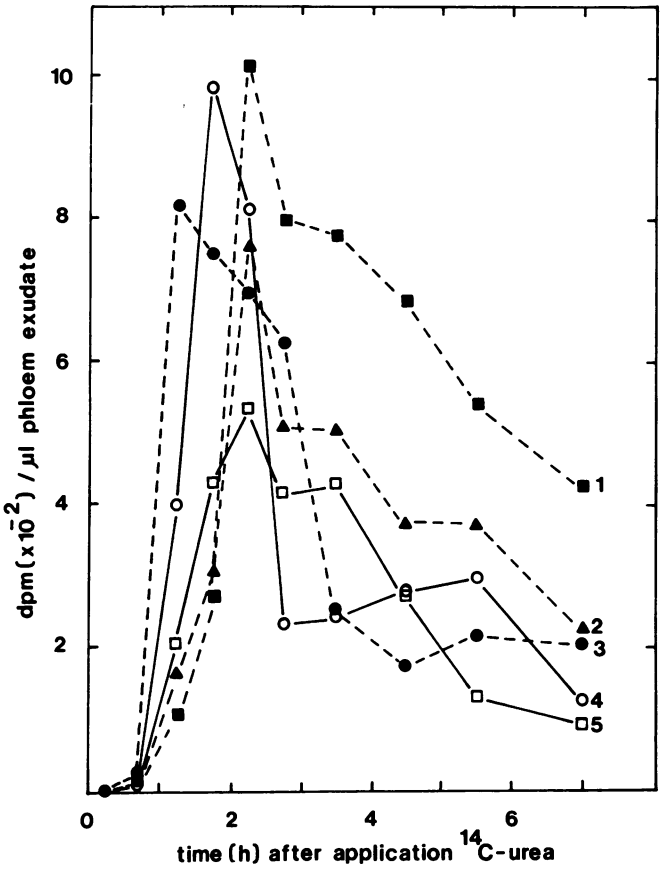

FIG. 2. Labeling of phloem exudate collected from sites of cryopuncture on fruits following surface application of [^{14}C]urea to the leaflets of the illuminated subtending leaf (L_4, see Fig. 1).

of the amide. Fruit phloem exudate labeling following feeding of [^{14}C]allantoin showed 50 to 54% of the label in sugar plus organic acids (mostly as labeled sucrose), indicating breakdown of the ureide through the ureolytic pathway (1) and reassimilation of the resulting $^{14}CO_2$ in shoot photosynthesis (see also data from earlier aphid-feeding studies [4]). With leaflet fed [^{14}C]allantoin, only 21% of the label in phloem remained as ureide and significant amounts of ^{14}C were present in the amino acids Asn, Gln, Ala, and Val, but, when fed through the cut shoot, ureides (46% of the label) were the only fraction to be labeled other than sugars plus organic acids. Negligible [^{14}]Curea was detected in phloem exudates following supply of [2-^{14}C]allantoin either in the transpiration stream or through the leaf reverse flap.

Experiments involving application of $^{14}CO_2$ to the fruit gas space or [^{14}C]urea to different sites on the outside surface of the pod (Fig. 1) provided conclusive evidence of transfer of assimilates generated in the pod tissues to phloem exudates obtained by cryopuncture of the dorsal vasculature of the fruit (Table V). [^{14}C]Urea feeding (Table V) indicated transfer from ventral to dorsal parts of the pod, presumably by means of the lateral veins, as well as proximal or distal transfer of label along the dorsal suture of the pod to the site of bleeding.

Fruit phloem sap collected over a period of 24 h after application of $^{15}NO_3$ (98 atom % excess ^{15}N) to the roots of intact plants showed a distribution of ^{15}N among amino compounds and in ureides as shown in Table VI. Nitrate was absent from the sap and 88% of the ^{15}N was recovered as eight amino compounds and ureides. Most of the ^{15}N was recovered as Asn (42%) and Gln (33%), with lesser amounts as Ala (3%) and His (3%). Compounds achieving particularly high atom % excess ^{15}N were Asn (10.2 atom % excess ^{15}N), Ala (8.6), and Gln (7.8). Ureide labeling was much lower (0.4 atom % excess) than that of all amino compounds, and well below that of the mean enrichment of the total N of the sap (6.1 atom % excess). The data emphasize the importance of amides in translocating N formed during NO_3 reduction in the vegetative parts of the shoot.

DISCUSSION

The exudate obtained following cryopuncture of the dorsal suture of attached cowpea fruits is considered to be a relatively pure exudate from phloem for the following reasons. It contains high levels of sugar (14 to 28%, w/v), with sucrose accounting for over 95% of total soluble sugars and for upwards of 79% of total C. The surrounding pod walls, by contrast, contain a mixture of sugars, principally monosaccharides (glucose and fructose), at lower concentration (2–3%, w/v, on a tissue water basis). Like phloem bleeding sap of other species, including legumes (8, 16, 31), protein is at a low level, nitrogenous compounds account for the second most abundant class of solutes after sugars, K^+ (950 to 1360 µg/ml) (24.3 to 34.8 µmol/ml) is the major inorganic cation, and sap levels of P and Mg^{2+} are high relative to those of Ca^{2+}. Nitrate is virtually absent from the phloem bleeding exudate despite its abundance in the xylem stream and in the walls of the pod close to the site of bleeding when plants are fed with this source of N. The exudate bleeding from the fruit is readily labeled with ^{14}C following application of [^{14}C]urea to the surface of the subtending leaf or to the pod wall of the bleeding fruit. Similarly application of [^{14}C]Asn and [^{14}C] allantoin to leaf or cut shoot through the xylem leads to phloem labeling in the fruit. The time course of labeling of the exudate

Table IV. *Distribution of Radiocarbon among Solutes of Fruit Phloem Exudate after Application of [U-^{14}C] Asparagine or [2-^{14}C]Allantoin through the Transpiration Stream or Blossom Leaflets of Cowpea*

Fruits were used for study 12 to 15 d after anthesis. Applications were made as 5 µCi of radiosubstrate to cut shoot through xylem, with 6-h collection period of fruit cryopuncture phloem sap (see xylem feeding, Fig. 1) for transpiration stream or applied as 3 µCi of radiosubstrate through the xylem of the midvein of the attached midleaflet of the leaf subtending the bleeding fruit (see reverse leaf flap feeding, Fig. 1) for blossom leaflets.

^{14}C-Labeled Substrate and Mode of Application	Distribution of ^{14}C among Labeled Solutes							
	Asp	Thr/Ser	Asn	Gln	Ala	Val	Ureides[a]	Sugars + organic acids
					%			
Asn, xylem fed	0.4		83.9	4.6				11.1
Asn, leaf flap fed	3.1	5.3	54.3	2.1	6.3			28.9
Allantoin, xylem fed							46.5	53.5
Allantoin, leaf flap fed			7.3	9.1	5.5	6.8	21.3	50.0

[a] Allantoin and allantoic acid.

Table V. *Transfer of ^{14}C of $^{14}CO_2$ Fed to the Fruit Gas Space or of [^{14}C]Urea Fed to Different Parts of the Pod Wall to Cryopuncture Phloem Exudate of the Mid-dorsal Suture of 15-d Attached Fruits of Cowpea*

Form and Site of Application of ^{14}C[a]	^{14}C Activity of Fruit Phloem Sap[b]
	dpm/μl
$^{14}CO_2$ to pod gas space	1817
[^{14}C]Urea to dorsal surface of pod, 2 cm proximal to puncture site	170
[^{14}C]Urea to dorsal surface of pod, 2 cm distal to puncture site	70
[^{14}C]Urea to ventral surface of pod, directly below	95

[a] All fruits fed 0.5 μCi ^{14}C (as $^{14}CO_2$ or [^{14}C]urea) while fully illuminated. Cryopuncture sap collected for a 5-h period. See Figure 1 for pictorial representation of feeding and collection sites.

[b] Mean activity of bulked sample of sap from two identically fed fruits.

Table VI. *Atom Per Cent Excess ^{15}N and Percentage Distribution of ^{15}N among Nitrogenous Solutes of Fruit Cryopunture Phloem Exudate of Plants fed $^{15}NO_3$ Via Their Roots*

Plants raised on unlabeled NO_3 (10 mM) and fed $^{15}NO_3$ (98 atom % excess ^{15}N, 10 mM NO_3) for a 24-h period during which phloem sap was collected continuously from their 12 to 15-d fruits.

Sap Constituent	N	Excess ^{15}N	Sap ^{15}N
	μg/ml	*atom %*	*%*
Ureide	220	0.43	0.8
Asparagine	515	10.23	42.2
Glutamine	536	7.76	33.3
Alanine	48	8.65	3.4
Threonine + serine	144	2.45	2.8
Valine	74	1.46	0.9
Isoleucine + leucine	111	1.93	1.7
Histidine	148	2.53	3.0
Total N	2050	6.10	100

after leaf feeding is similar to that of aphids collected from the fruit. In either case, a translocation rate for the front of labeled solutes of 21 to 38 cm/h is indicated (see similar values for phloem translocation in other studies [6]). The high intensity of labeling suggests significant mass transfer of the ^{14}C of the applied substrate and products of its metabolism to the puncture site. The prompt appearance of ^{14}C in exudate after feeding $^{14}CO_2$ or [^{14}C]urea to adjacent parts of an illuminated fruit indicates that locally produced photoassimilates may contribute to phloem loading in addition to photosynthate and xylem-borne solutes originating in other parts of the plant.

Despite the preliminary nature of the present observations, the labeling studies and comparisons of relative amounts of nitrogenous solutes in xylem root bleeding sap and phloem-cryopuncture fruit exudate of NO_3-fed and symbiotic (−N) plants, suggest several interesting features relating to the assimilation and translocation processes for N in cowpea. Confirming earlier studies of xylem transport in this and other ureide-producing legumes (3, 17, 20), utilization of NO_3 is characterized by high (78%) "spill-over" of free NO_3^- to the shoot from the root, low ureide transport from roots relative to amides and amino acids, and a high ratio of Asn:Gln in xylem sap. By contrast, the xylem transport of plants using symbiotically fixed N shows a marked preponderance of ureide-N over amino N and a high ratio of Gln:Asn. The matching phloem analyses suggest that these substantially different proportions of solutes supplied from roots in the transpiration stream are processed in the shoot in such a manner that the resulting phloem streams to the fruit carry remarkably similar levels and proportions of nitrogenous solutes in NO_3-fed and nodule-fed plants. Thus, as shown in the ^{15}N-feeding studies, the NO_3^- of xylem is not available for phloem bleeding, but is reduced in leaf and stem (3) and the reduction products, principally Asn and Gln, loaded onto the phloem. In similar fashion, shoots of symbiotically active plants augment the level of amino N in the phloem stream serving their fruits by utilizing ureide supplied in the xylem as a source of N for synthesis of amino compounds in leaves. This is suggested in the present [^{14}C]allantoin-feeding studies, and in the earlier labeling studies using aphids (4).

The cryopuncture technique offers prospects for more detailed studies of the nutritional economy of cowpea fruits, along the lines conducted for *P. sativum* (19), *L. albus* (12, 22), and *Glycine max* (14). Direct collection of phloem exudates allows direct measurements of concentrations of phloem-borne materials and hence of the likely amounts of water entering the fruit in phloem. The technique has the added advantage that in labeling studies metabolic changes during or after collection from the phloem are eliminated or at least minimized. These are likely to occur with either the EDTA technique (7, 13, 26, 28) or when the bodies of aphids feeding on phloem are used to collect phloem-borne assimilates (4).

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