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COMPARATIVE BIOCHEMISTRY OF THE LYCOPODS

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Abstract—The Lycopodiales are shown to differ from the Selaginellales in two important respects: (1) lignin composition and (2) sugars produced in photosynthesis. *Selaginella* species contain angiospermous lignins with syringyl units predominating whereas *Lycopodium* and *Phylloglossum* appear to have a gymnospermous type of lignin. The major sugar formed in photosynthesis is trehalose in *Selaginella* and sucrose in *Lycopodium*. Species of *Isoetes* have also been included in these studies.

INTRODUCTION

THE five living genera of the Lycopodophyta are usually placed in three orders: Lycopodiales (*Lycopodium* and *Phylloglossum*), Selaginellales (*Selaginella*) and Isoetales (*Isoetes* and *Stylites*). These are remnants of a group of plants whose history extends back at least to the Devonian.[1] Although they share many important characteristics with each other and with certain fossil genera, the extant lycopods also display marked morphological differences between each other, differences which appear to have arisen relatively early during the long period of their evolutionary history. It seems reasonable to suppose that the morphological differences are reflected in the biochemistry of these botanically interesting plants.

Previous work[2, 3, 4] has indicated that *Lycopodium* has a gymnospermous type of lignin. Alkaline oxidation of the lignin of a number of species yields vanillin and *p*-hydroxybenzaldehyde but not syringaldehyde. Some species, however, yield syringic acid as well as guaiacyl compounds and in this respect their lignin is different from that of either gymnosperms or angiosperms. *Phylloglossum*, the only other genus in the Lycopodiales, is similar to *Lycopodium*.[5] *Selaginella* and *Isoetes* are reported[2] to have essentially an angiospermous type of lignin, i.e. syringaldehyde as well as vanillin and *p*-hydroxybenzaldehyde are obtained on lignin oxidation. Quantitative data, presented in this paper, confirm that *Selaginella* lignin resembles that obtained from angiosperms.

Trehalose, a relatively rare sugar in green land plants, has been isolated from twenty-one species of *Selaginella*.[6] *Lycopodium*, on the other hand, is known to contain sucrose.[4] This aspect of lycopod biochemistry has been examined in greater detail in the present study.

RESULTS AND DISCUSSION

Phenolic Acids and Lignins

Alkaline hydrolysates of ethanolic extracts of all species of *Selaginella* examined were found to contain syringic acid in addition to vanillic *p*-hydroxybenzoic, *p*-coumaric, ferulic

[1] J. WALTON, *An Introduction to the Study of Fossil Plants*. Adams & Chas. Black, London (1953).
[2] G. H. N. TOWERS and R. D. GIBBS, *Nature* **172**, 25 (1953).
[3] K. KRATZL and J. EIBL, *Intern. Holzmarkt, Wien* 25, *Mitteilung der Ogh*, 77 (1951).
[4] G. H. N. TOWERS and W. S. G. MAAS, *Phytochem.* **4**, 57 (1964).
[5] AIDA TSE, ELEANOR WHITE and G. H. N. TOWERS, *Nature* (In press).
[6] T. YAMASHITO and F. SATO, *J. Pharm. Soc. Japan* **49**, 696 (1929).

and caffeic acids. In contrast to this, only certain species of *Lycopodium* yielded syringic acid.[4] Quantitative analyses of the substituted benzaldehydes obtained on alkaline copper hydroxide oxidation of pre-extracted wood-meals of species of *Selaginella* are shown in Table 1. Although this represents a relatively small sampling of the genus (11 out of a possible 700 species), the consistent yield of syringaldehyde on oxidation is noteworthy. The generally high ratio of syringaldehyde to vanillin, moreover, is reminiscent of angiosperm lignin. This is in direct contrast to *Lycopodium* lignins which have never been shown to yield syringaldehyde. If angiospermous lignin is considered to be a more complex polymer or mixture of polymers than gymnospermous lignins then *Selaginella* appears to possess an advanced biochemical feature over *Lycopodium*. Since tyrosine gives good yields of *p*-hydroxybenzaldehyde on alkaline oxidation it is possible that some, if not all, of this aldehyde shown in Table 1 is derived from protein tyrosine. This possibility was not examined.

TABLE 1. YIELDS OF ALDEHYDES ON ALKALINE CUPRIC HYDROXIDE OXIDATIONS OF PRE-EXTRACTED WOOD-MEALS OF *Selaginella* spp.

Species	Wood-meal oxidized (mg)	Syringaldehyde (mg)	Vanillin (mg)	*p*-Hydroxybenzaldehyde (mg)
S. pallescens "Emiliana" (Presl) Spring	900	3·23	0·63	0·60
S. pallescens "nobilis" (Presl) Spring	946	2·51	0·91	1·11
S. martensii Spring	996	3·88	1·60	1·73
S. caulescens (Wall.) Spring	900	4·69	1·80	1·00
S. grandis Moore	635	0·65	0·26	0·19
S. wildenowii (Desv.) Baker	840	1·45	0·31	0·53
S. pulcherrima Liebm.	904	2·83	1·65	1·45
S. umbrosa Lemaire ex Hieron	900	0·47	0·11	0·43
S. helvetica (L.) Link	893	3·36	1·42	1·67
*S. elegans**	857	0·39	0·30	0·38
*S. stenophylla**	810	4·25	1·41	0·79

* The authorities for these names are not available to us at present.

Although *Isoetes* is reported to yield both vanillin and syringaldehyde[2] the very small amounts of lignin in the small samples of plant material available precluded a quantitative study.

Sugars

Chromatographic analyses of the neutral fraction indicated that species of *Selaginella* contain scurose, glucose and fructose in addition to trehalose although the last sugar is usually the major one present. *Selaginella pallescens* for example, contains readily detectable amounts of sucrose in addition to trehalose. Evidence for the presence of trehalose was obtained with some species of *Lycopodium* although, in all species examined, sucrose was the predominant sugar. In view of the presence of both sucrose and trehalose in some species of *Selaginella* and *Lycopodium* this chemical characteristic, i.e. type of sugar reserve, would appear to have little value for taxonomic purposes.

An interesting state of affairs was revealed, however, in a more dynamic approach to this problem. It was found that in relatively short periods of photosynthesis in $^{14}CO_2$, trehalose was the predominantly labelled sugar in *Selaginella* whereas most of the activity in the sugar

fraction of *Lycopodium* was in sucrose. In *Lycopodium* no radioactivity appeared in the region of trehalose on chromatograms. Table 2 shows the results of these experiments. Plants collected from different localities showed a consistent behaviour with respect to the sugars synthesized. *Isoetes*, showed a somewhat similar pattern to *Lycopodium* except for the fact that trehalose as well as sucrose was radioactive in a 2 hr period of photosynthesis.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN THE NEUTRAL AQUEOUS EXTRACT OF PLANTS AFTER PHOTOSYNTHESIS IN $^{14}CO_2$

Species	Source	Total radioactivity in the fraction (μc)	% Radioactivity*				
			Trehalose	Sucrose	Glucose	Fructose	Other
Selaginella wallacei Hieron	Horseshoe Bay, B.C.	12·1	91	8	0·5	0·5	—
	Sooke, B.C.	21·4	88	10	0·2	0·5	1·3
	Oregon, U.S.A.	19·3	81	18	0·3	—	0·7
	Tofino, B.C.	21·9	98	1	0·2	—	0·8
S. kraussiana (Kze.) A. Br.	Tofino, B.C.	18·8	42	1	0·2	0·2	56†
S. densa Rydb.	Kootenay Lake	21·2	96	3	0·4	0·1	0·5
	Kootenay Lake	19·6	95	1	0·2	1·7	2·1
Isoetes bolanderi Engelm.	Kennedy Lake—2 hr	0·6	4	77	3	3	13
	Kennedy Lake—5 hr	4·8	7	63	2	2	26
I. occidentalis Henderson	Marion Lake—2 hr	1·2	8	64	2	2	24
	Marion Lake—5 hr	1·4	17	40	2	2	39
Lycopodium clavatum L.	Parksville	19·5	—	96	1	1	2
	Tofino, bog	23·5	—	96	2	1	1
	Tofino, mountain	19·8	—	97	1	0·4	1·6
	Squamish	16·2	—	98	0·6	0·7	0·7
L. selago (L.) Bernn.	Nanaimo	18·8	—	97	0·5	0·5	2
	Tofino	19·1	—	96	0·5	0·5	3
L. sitchense Rupr.	Garibaldi	6·6	—	98	0·8	0·8	0·3
	Mt. Seymour	15·4	—	98	0·5	0·5	1
L. annotinum L.	Bowron Lakes	18·6	—	96	1	1	2
	Prince George	19·0	—	98	0·5	0·5	1
	White River	20·1	—	98	—	0·5	1·5
L. complanatum L.	White River	21·9	—	98	0·5	0·5	1
L. obscurum L.	Bowron Lakes	18·2	—	95	0·7	0·3	4
Botrychium virginianum (L.) Sm.	Parksville	17·8	—	82	8	0·5	9·5
	White River	19·5	11	63	10	1	15

* As per cent of radioactivity in the compounds excised from the chromatogram. These were all but the faintest spots that darkened the chromatograms, and represented at least 99·5 per cent of the total radioactivity on the chromatograms.

† Fifty-five per cent of the radioactivity in the neutral fraction of *S. kraussiana* occurred in a spot which chromatographed nearer to the origin than trehalose.

The fact that, among the vascular cryptogams, trehalose synthesis is not confined to *Selaginella* or *Isoetes* is indicated by the work of Kandler and Senser[7] and by our inclusion of a fern in the present study. Kandler and Senser have demonstrated that, after 30 min photosynthesis in $^{14}CO_2$, 20 per cent of the radioactivity in *Botrychium lunaria* is in trehalose. We were able to demonstrate trehalose synthesis from $^{14}CO_2$ in one sample of *B. virginianum*

[7] D. KANDLER and N. SENSER, *Z. Pflanzen Physiol. Z. Bot.* **53**, 157 (1965).

but not in another from a different locality (see Table 2). *Botrychium* is a relatively primitive fern and a thorough examination of the situation with respect to sugar synthesis in the ferns seems warranted.

Analyses of the amino acid fraction obtained in the experiments with *Lycopodium* and *Selaginella* showed no regular pattern. In some cases differences between species of *Lycopodium* or between species of *Selaginella* were much greater than overall differences between the genera. No basic distinction could be made between *Lycopodium*, *Selaginella* or *Isoetes* nor were there any unusual amino acids detectable on chromatograms or radioautographs. The amount of radioactivity in this fraction was usually one-fifth to one-twentieth of that of the neutral fraction.

Radioactive trehalose, isolated from *S. wallacei*, which had assimilated $^{14}CO_2$, was administered to a few representative species. The results, shown in Table 3 indicate that trehalose is utilized in the synthesis of sucrose by all three genera. This may be achieved in one of the following ways:

(1) $$\text{Trehalose} \xrightarrow{H_2O} \text{glucose} \xrightarrow{ATP} \xrightarrow{UTP} \text{UDPG} \xrightarrow{\text{Fructose}} \text{sucrose}$$

(2) $$\text{Trehalose} \xrightarrow{UDP} \text{UDPG} \xrightarrow{\text{Fructose}} \text{sucrose.}$$

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN TREHALOSE, SUCROSE, GLUCOSE AND FRUCTOSE FROM PLANTS ADMINISTERED TREHALOSE-^{14}C

Species	Total radioactivity in aqueous neutral fraction (μc)	% Radioactivity*				
		Trehalose	Sucrose	Glucose	Fructose	Origin
Lycopodium selago	0·057	32	38	22	6	2
L. obscurum	0·078	14	48	29	7	3
Selaginella emmeliana	0·180	23	58	14	2	3
Isoetes bolanderi	0·129	79	9	9	2	1
Tmesipteris tannensis	0·070	32	37	24	4	3

* As per cent of radioactivity in the compounds excised from the chromatogram.

Ability to utilize trehalose for the synthesis of sucrose is also a feature of *Tmesipteris*, an interesting and relatively rare cryptogam which was included in this study since a sample of living material was available at the time of the experiments.

An important conclusion of this study is that the rates of formation of particular compounds in photosynthesis from $^{14}CO_2$ may be of value in determining biochemical similarities or differences between groups of plants. Thus even relatively common metabolites such as sucrose and trehalose may be useful as markers of taxonomic affinities. We suggest that this method could be extended to other classes of compounds such as the alkaloids and terpenoids to provide valuable information in chemotaxonomic studies. This approach may be necessary especially in those cases where chemical differences in plants are of a quantitative rather than a qualitative nature.

Further chemical and biochemical studies of lycopods appear to be warranted. For example, amentoflavone and sotetsuflavone have been reported to be constituents of *S. tamariscina*[8] and a search for these biflavonyls should be extended to other lycopods. The

[8] H. Y. HSU, *Bull. Taiwan Prov. Hyg. Lab.* No. 1 (1959).

results of surveys of this nature may throw more light on the varied patterns of metabolism which have become established in these three closely related orders of non-flowering plants.

METHODS

Voucher specimens of all plant materials used in these analyses, have been retained.
Methods for the determination of phenolic acids and lignins have been described.[4]

Administration of $^{14}CO_2$ in Photosynthesis and Analysis of Extracts for Sugars and Amino Acids

The apparatus used was essentially that described by Bidwell.[9] The photosynthetic chamber was illuminated with approximately 10,000 lx and whole plants, with their roots in water, were used. In each case about 20 g plant material was administered 50 μc of $^{14}CO_2$ for 2 hr unless otherwise indicated.

At the end of the experiment the plant material was ground in a Waring Blendor in boiling 80% ethanol. The residue obtained by evaporating the ethanolic extract was suspended in 15 ml chloroform and 25 ml water and the two phases separated by centrifugation. The aqueous layer was passed through a column of 60 ml of Rexyn 101 (organic, strong acid cation exchanger obtained from Fisher Scientific Company). The effluent was passed on to a column of 50 ml of Dowex 3 (weakly basic anion exchanger, J. T. Baker Chemical Co.). The columns were washed with about 250 ml distilled water, the effluent from the cation exchanger passing on to the anion exchanger. The final effluent comprised the neutral fraction. The cation exchanger was next treated with 250 ml 1 M NH_4OH to give an eluate containing the amino acids.

Two-directional chromatograms of the neutral and the amino acid fraction were prepared using Whatman No. 1 chromatography grade paper and phenol/water/conc. NH_4OH (80:19·7:0·3) for the first direction and *n*-propanol/ethyl acetate/water (7:1:2) for the second. Sugars were detected using various spray reagents particularly that of Trevelayn *et al.*[10] Amino acids were detected with ninhydrin.

Methods of radioautography were essentially those described by Ibrahim and Towers.[11] All radioactivity measurements were made with a Nuclear-Chicago Liquid Scintillation Spectrometer and were corrected for background and efficiency. To measure the radioactivity of spots excised from paper chromatograms a solution consisting of 4 g PPO, 50 mg POPOP and toluene to make 1 l., was used.

Isolation of Radioactive Trehalose

Because of limitations in the size of the photosynthetic chamber, small samples (about 10 g) of *Selaginella wallacei* were allowed to photosynthesize in $^{14}CO_2$ (30 μc) for 2 hr until a total of approximately 100 g of plants had been fed 300 μc of ^{14}C. Attempts to isolate trehalose by the method of Yamashito and Sato[6] were unsuccessful. Isolation was achieved finally by banding the de-ionized extract on sixty sheets of Whatman No. 1 paper and chromatographing in the propanol/ethyl/acetate/water solvent. Trehalose was eluted from the chromatogram with about 2 l. of water, the eluate was concentrated, decolorized by boiling with charcoal and crystallized from aqueous ethanol. About 1 g of large crystals was obtained with an i.r. spectrum identical to that of authentic trehalose and with an activity of 0·143 μc/mg.

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[9] R. G. S. Bidwell, *Can. J. Botany* **36**, 337 (1958).
[10] W. E. Trevalayn, D. P. Procter and J. S. Harrison, *Nature* **166**, 444 (1950).
[11] R. K. Ibrahim and G. H. N. Towers, *Nature* **184**, 1803 (1959).