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Studies on the Water Soluble Constituents of Lichens. I. Gas Chromatographic Analysis of Low Molecular Weight Carbohydrates. (1)[1]

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The low molecular weight carbohydrate constituents of eight species of lichens (*Cetraria islandica*, *Cladonia crispata*, *C. rangiferina*, *C. squamosa*, *Peltigera aphthosa*, *P. nigripunctata*, *Umbilicaria caroliniana*, and *Usnea rubescens*) have been analysed as their acetyl, trifluoroacetyl, and trimethylsilyl derivatives by gas chromatography. Glycerol, erythritol, ribitol, arabinitol, mannitol, fructose, glucose, sucrose, and trehalose were detected commonly in these lichens. In addition, the occurrence of 3-O-β-D-glucopyranosyl-D-mannitol in *P. aphthosa* and *P. nigripunctata* and of 2-O-β-D-galactofuranosyl-D-arabinitol (umbilicin) in *C. islandica* and *U. caroliniana* have been found.

So far comparatively little work has been done on the distribution of the low molecular weight carbohydrates in lichens.[3,4] However, it has generally been recognized that in lichens various polyols comprise the predominant carbohydrates and free sugars are not normally found as abundant constituents. In addition, some lichens are known to contain characteristic glycosides of sugar alcohols.

On the other hand, Smith and his co-workers have recently developed the extensive investigations on the carbohydrate metabolism in lichens and indicated that the identity of the carbohydrate moving between the symbionts of a lichen depends on the genus of alga present.[3,5] For example, in lichens containing five genera of Chlorophyceae carbohydrates move to fungus as polyols (ribitol in *Trebouxia*, *Myrmecia*, and *Coccomyxa*; erythritol in *Trentepholia*; and sorbitol in *Hyalococcus*), and in lichens possessing three genera of Cyanophyceae (*Nostoc*, *Calothrix*, and *Scytonema*) glucose is the carbohydrate moving. Heretofore, however, the occurrence of the carbohydrates mentioned by Smith has been demonstrated only in a limited number of lichens.[3,4]

In earlier studies, most lichens have been analysed by paper chromatography or by paper electrophoresis. But both the methods are not suitable for the detection of such carbohydrates as polyols, polyol-glycosides, and non-reducing disaccharides, especially when they occur in low concentrations, since the usual reagents are not sensitive to them. Thus, it is probable that previous workers have overlooked the presence of certain minor constituents. Gas chromatography has now become available for the identification and quantification of the carbohydrates in plant tissues,[6] nevertheless the technique has little been utilized as yet in the field of lichens.[7,8]

1) This work was presented at the 92nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April, 1972.
2) Location: *13-1 Takara-machi, Kanazawa.*
3) D.H. Lewis and D.C. Smith, *New Phytol.*, **66**, 143 (1967).
4) C.F. Culberson, "Chemical and Botanical Guide to Lichen Products," The University of North Carolina Press, Chapel Hill, 1969.
5) D.H.S. Richardson, D.J. Hill, and D.C. Smith, *New Phytol.*, **67**, 61, 69, 469 (1968); E.A. Drew and D.C. Smith, *ibid.*, **66**, 379, 389 (1967); D.H.S. Richardson, D.C. Smith, and D.H. Lewis, *Nature*, **214**, 879 (1967); E.A. Drew and D.C. Smith, *Lichenologist*, **3**, 197 (1966); D.H.S. Richardson and D.C. Smith, *ibid.*, **3**, 202 (1966).
6) P.M. Holligan, *New Phytol.*, **70**, 239 (1971).
7) P.M. Holligan and E.A. Drew, *New Phytol.*, **70**, 271 (1971).
8) T. Komiya and S. Shibata, *Phytochem.*, **10**, 695 (1971).

The present paper deals with the gas chromatographic analyses of low molecular weight carbohydrates in the following eight species of lichens: *Cetraria islandica* (L.) Ach., *Cladonia crispata* (Ach.) Flot., *C. rangiferina* (L.) Web. ex Wigg., *C. squamosa* (Scop.) Hoffm., *Peltigera aphthosa* (L.) Willd., *P. nigripunctata* Bitt., *Umbilicaria caroliniana* Tuck., and *Usnea rubescens* Stirt. Of the lichens examined, three species, *i.e.*, *C. islandica*,[9] *C. rangiferina*,[9,10] and *P. aphthosa*.[7,9,11] have already been reported on their carbohydrate constituents. The previous results are compared with our present data in the discussion part.

The methanol extracts of the lichens were worked up as described in the experimental part and the resulting carbohydrate fractions were analysed as their acetyl (Ac), trifluoroacetyl (TFA), and trimethylsilyl (TMS) derivatives on the columns of SE-30, XF-1105, and OV-17, respectively, with temperature programmed operation.

Experimental

Lichens——*Cladonia crispata*, *C. rangiferina*, and *C. squamosa* were all collected in Kumogahata, Kyoto, in Sept., 1965. *Peltigera aphthosa* was collected in Mt. Fuji, in June, 1967. *P. nigripunctata* was collected around Lake Syozi, in Dec., 1966. *Umbilicaria caroliniana* was collected in Mt. Kinpu, in July, 1965. *Usnea rubescens* was collected in the suburbs of Sendai, in May, 1967. *Cetraria islandica* was commercially obtained from Merck and Co. many years ago, and the habitat and date of collection were not recorded. Until use, these lichens had been stored at room temperature.

Preparation of the Carbohydrate Fraction——The lichens were extracted thrice by refluxing with MeOH, and the extracts were concentrated *in vacuo* to dryness. The residue was partitioned between $CHCl_3$ and H_2O, then the aqueous phase was treated with basic lead acetate. After centrifugation, excess lead in the supernatant was precipitated by H_2S. The filtrate was passed through the columns of Amberlites IR-120 (H^+) and IRA-400 (OH^-) and the unadsorbed effluent was concentrated *in vacuo* to give a syrup, which was azeotroped with benzene, repeatedly, and dried over P_2O_5. The yields of the resulting carbohydrate fractions are shown in Table II.

Standard Carbohydrates——The following carbohydrates were purchased from the commercial sources and were of guaranteed grade purity: glycerol, erythritol, mannitol, sorbitol, myo-inositol, arabinose, xylose, fructose, galactose, glucose, sucrose, and trehalose. Ribitol, rhamnitol, arabinitol, and galactitol were prepared from the corresponding sugars by reduction with $NaBH_4$. The standard specimens of the polyol-glycosides were obtained as described below.

Isolation of 3-O-β-D-Glucopyranosyl-D-mannitol from *P. nigripunctata*——The carbohydrate fraction of *P. nigripunctata* was acetylated and the resulting mixture was chromatographed on silica gel with hexane–AcOEt (2: 1) as eluant. The acetates of polyols and of monosaccharides were first eluted. Successive elution with the same solvent afforded a fraction, as semi-solid, whose gas chromatogram consisted almost solely of a peak. The peak corresponded to the major peak in the disaccharide region of the gas chromatogram shown by the starting mixture. Presence of the identical peak was also observed in the gas chromatogram of the acetylated carbohydrate fraction prepared from *P. aphthosa* in which occurrence of 3-O-β-D-glucopyranosyl-D-mannitol had been reported.[11] The fraction was hydrolysed with 1N HCl to give glucose and mannitol in a ratio of 1: 1.

Isolation of Umbilicin (2-O-β-D-Galactofuranosyl-D-arabinitol) from *U. caroliniana*——The carbohydrate fraction of the lichen was acetylated and worked up as above to give a fraction, which showed a peak corresponding to the largest peak in the gas chromatogram of the original mixture. The presence of the identical peak could be detected in the gas chromatogram of the acetylated carbohydrate fraction prepared from *C. islandica*, which had been known to contain umbilicin.[9] On acid hydrolysis the fraction yielded arabinitol and galactose (1: 1).

Preparation of the Volatile Derivatives——Acetylation: About 2 mg of the sample was acetylated with Ac_2O and pyridine (1 ml, each) at room temperature for 12 hr. The reaction mixture was poured into ice-water and extracted with $CHCl_3$. After concentration, the $CHCl_3$ solution was injected into the gas chromatograph. Trifluoroacetylation: This was performed by Tamura's method.[12] The well-dried sample (1 mg) was treated with N,N-dimethylformamide (0.1 ml) and trifluoroacetic anhydride (0.2 ml) at room

9) B. Lindberg, A. Misiorny, and C.A. Wachtmeister, *Acta Chem. Scand.*, **7**, 591 (1953).
10) G. Pueyo, *Bryologist*, **68**, 334 (1965).
11) *a*) B. Lindberg, B.-G. Silvander, and C.A. Wachtmeister, *Acta Chem. Scand.*, **17**, 1348 (1963); *b*) *Idem*, *ibid.*, **18**, 213 (1964).
12) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 2314 (1970).

temperature for 10 min, and an aliquot of the reaction mixture was injected directly into the gas chromatograph. Trimethylsilylation: The well-known silylation method of Sweeley, *et al.* was adopted.[13]

Gas Chromatographic Conditions——A Shimadzu GC-4BPF dual column analytical gas chromatograph with a hydrogen flame ionization detector and U-shaped glass columns were used. Nitrogen was employed as carrier gas at the flow rate of 50 ml/min. Other conditions adopted for analyses of the respective derivatives were as follows: Ac Derivatives: Column packing, 1.5% SE-30 on Chromosorb W (AW–DMCS) (60—80 mesh); tube size, 2.0 m×4 mm *i.d.*; temperature programme, 6 min isothermal hold at 180° followed by 6°/min linear increase to 250°. TFA Derivatives: Column packing, 1.0% XF-1105 on Chromosorb W (AW–DMCS) (60—80 mesh); tube size, 1.5 m×4 mm *i.d.*; temperature programme, 10 min isothermal hold at 140° followed by 5°/min linear increase to 200°. TMS Derivatives: Column packing, 1.5% OV-17 on Shimalite W (AW) (80—100 mesh); tube size, 2 m×4 mm *i.d.*; temperature programme, 20 min isothermal hold at 160° followed by 5°/min linear increase to 250°.

Identification of the Carbohydrate Constituents——Peak identification was based on the comparison of three relative retention times for Ac, TFA, and TMS derivatives of each component with those for the corresponding derivatives of the respective standard carbohydrate, followed by co-chromatography to obtain coincident peaks on the chromatogram. The relative retention times are listed in Table I and some representative gas chromatograms are shown in Fig. 2.

Quantitative Estimation——Most carbohydrates were quantitatively analysed as their TFA derivatives. But in the cases of glycerol and the polyol-glycosides, Ac derivatives were employed. Peak areas were determined as peak height measurements, throughout the present work. As shown in Fig. 1, the calibration curves for respective carbohydrates were prepared with good linearity by using the standard specimens. The relative response factors thus obtained were as follows: a) As TFA Derivatives: Erythritol, 3.77; ribitol, 2.10; arabinitol, 1.37; mannitol (standard), 1.00; fructose, 0.75; glucose, 0.65; sucrose, 0.21; and trehalose, 0.20. b) As Ac Derivatives: glycerol, 4.55; mannitol (standard), 1.00; the mannitol-glucoside, 0.29; and umbilicin, 0.34. By applying these values the respective proportions of the carbohydrate constituents in the sample were calculated, and all estimates were expressed as percentages of the total dry weight of the carbohydrate fraction (Table II). The absolute contents of the respective carbohydrates in the lichens can be calculated by multiplying these values by another data mentioned in Table II as the yields of the carbohydrate fractions. Thus internal standard was not used in the present study.

Table I. Relative Retention Times for Ac, TFA, and TMS Derivatives

Carbohydrate	Ac[a]	TFA[a]	TMS[a]
Glycerol	0.11	—	0.19
Erythritol	0.21	0.26	0.24
Arabinitol	0.51	0.65	0.46
Ribitol	0.48	0.53	0.46
Mannitol	1.00[b]	1.00[c]	1.00[d]
Fructose	0.82	0.72	0.67
			1.04
Glucose	0.85	0.72	1.17
		1.24	1.78
Sucrose	2.54	5.09	3.91
Trehalose	2.60	5.50	4.00
Mannitol-glucoside	2.91	6.00	4.07
Umbilicin	2.42	4.51	3.69
cf.[e]			
Xylitol	0.55	0.73	0.44
Galactitol	1.01	1.44	1.04
Sorbitol	1.07	1.14	1.07
Myo-inositol	0.98	1.82	1.84

a) Gas chromatographic conditions are described in the experimental part.
b) 9.8 min, *c*) 4.8 min, *d*) 8.6 min,
e) These carbohydrates were not detected from the lichens examined.

13) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

Result and Discussion

Table I shows the relative retention times for the Ac, TFA, and TMS derivatives of all the carbohydrates detected in the present lichens. The results indicate that the best separations can be achieved with TFA derivatives. Analyses as Ac derivatives resulted in the poor resolutions between arabinitol and ribitol, and also between fructose and glucose. As TMS derivatives, arabinitol was separable from ribitol but only partially. Therefore, quantitative estimation of most carbohydrates was performed by using their TFA derivatives. However, glycerol and the polyol-glycosides were determined as their Ac derivatives, since the peak of TFA glycerol showed a tendency to be masked by a solvent peak, and the standard specimens of the latter were isolated in the form of acetate as described in the experimental part. The calibration curves showed good linearity as illustrated in Fig. 1. The carbohydrate compositions of the lichens examined are summarized in Table II, and some representative gas chromatograms are depicted in Fig. 2.

Table II. Carbohydrate Compositions[a)]

Name of species	Dry wt. g	Carbohydrate fr. g(%)	Glycerol	Erythritol	Ribitol	Arabinitol
Cetraria islandica	5	0.1(2.0)	16.8	3.7	6.6	39.2
Cladonia crispata[c)]	90	1.6(1.8)	1.1	t[b)]	6.9	65.7
C. rangiferina[c)]	90	0.7(0.8)	4.3	t	9.2	66.0
C. squamosa	1290	3.6(0.3)	4.7	0.5	1.4	48.4
Peltigera aphthosa[c)]	420	16.7(4.0)	1.6	2.7	0.5	11.6
P. nigripunctata	410	13.4(3.3)	1.6	t	2.2	56.2
Umbilicaria caroliniana[c)]	25	0.4(1.6)	1.6	t	2.0	17.4
Usnea rubescens	119	3.7(3.1)	1.1	t	21.4	64.4

Name of species	Mannitol	Fructose	Glucose	Sucrose	Trehalose	Umbilicin	Mannitol-glucoside
Cetraria islandica	15.3	6.0	4.6	4.1	2.9	0.8	—[b)]
Cladonia crispata[c)]	16.9	6.9	2.5	—	—	—	—
C. rangiferina[c)]	5.8	13.9	0.8	t	t	—	—
C. squamosa	24.4	8.0	2.8	9.8	—	—	—
Peltigera aphthosa[c)]	21.2	0.6	1.2	t	t	—	60.5
P. nigripunctata	25.3	1.4	1.6	t	t	—	11.6
Umbilicaria caroliniana[c)]	14.8	1.3	2.7	t	t	60.2	—
Usnea rubescens	4.3	8.7	t	t	—	—	—

unidentified peaks:[d)] retention times relative to mannitol as TFA derivatives
C. islandica: 1.33, 2.55, 3.16, 3.57, 4.04 — *P. aphthosa*: 2.63, 3.88
C. crispata: 2.29 — *P. nigripunctata*: 2.66, 5.43
C. rangiferina: 0.86 — *U. caroliniana*: 1.58, 1.79, 2.58, 3.67
C. squamosa: 1.86, 2.68, 3.42, 3.84 — *U. rubescens*: 1.53
a) % by weight in carbohydrate fraction. *b*) t: trace, —: not detected *c*) Chromatogram is shown in Fig. 2.
d) All of these peaks are very small.

As can be seen in Table II, of eleven carbohydrates identified in the present study, seven, *e.g.*, glycerol, erythritol, ribitol, arabinitol, mannitol, fructose, and glucose were detected in all the lichens analysed, and two, *e.g.*, sucrose and trehalose, in almost all species. Besides them, two polyol-glycosides, *e.g.*, umbilicin[7,9,14)] and the mannitol-glucoside,[7,11,15)] were found to occur, individually, in some special lichens.

It has already been revealed by a number of authors that mannitol is universally distributed in various lichens and arabinitol is widespread in the lichens of Gymnocarpeae (but

14) H.F.G. Beving, H.B. Boren, and P.J. Garegg, *Acta Chem. Scand.*, **22**, 193 (1968); B. Lindberg and B. Wickberg, *ibid.*, **16**, 2240 (1962); *idem*, *ibid.*, **8**, 821 (1954); *idem*, *ibid.*, **7**, 140 (1953); *idem*, *ibid.*, **6**, 1052 (1952).
15) G. Pueyo, *Compt. Rend.*, **248**, 2788 (1959).

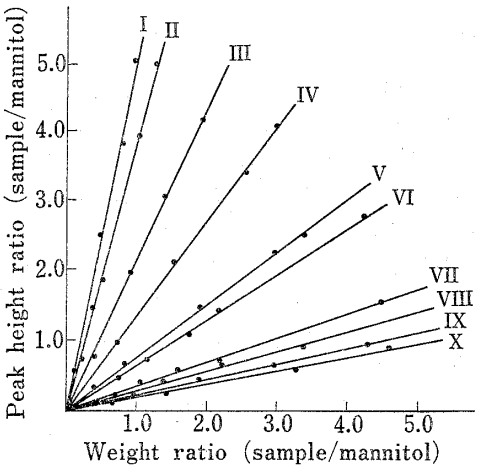

Fig. 1. Calibration Curves

I: glycerol (Ac), II: erythritol (TFA), III: ribitol (TFA), IV: arabinitol (TFA), V: fructose (TFA), VI: glucose (TFA), VII: umbilicin (Ac), VIII: mannitol-glucoside (Ac), IX: sucrose (TFA), X: trehalose (TFA)
Gas chromatographic conditions are shown in the experimental part.

not of Pyrenocarpeae).[3,4,9] Recently it has also been indicated by Smith and his co-workers that the carbohydrates, such as ribitol and glucose, photosynthesized in algal partners are released into the fungal partners where they are converted to mannitol and arabinitol.[5] Therefore, it is not surprising that the present lichen, all belonging to the order Gymnocarpeae, contained both the polyols in appreciable amounts. Previously, little has been reported of the quantitative comparison between these two polyols in a lichen. Thus it may be noteworthy to mention that arabinitol contents are larger than mannitol contents in seven out of eight species analysed by us. Other polyols and sugars commonly encountered in the present study are the constituents whose occurrence had been formerly demonstrated only in a few lichens. Our present results suggest that these carbohydrates will prove much more widespread than have previously been supposed. Their contents were, in general, very low, but there exist some exceptions. For example, content of glycerol in *C. islandica*, ribitol in *U. rubescens*, fructose in *C. rangiferina*, and sucrose in *C. squamosa* were found to be comparatively higher.

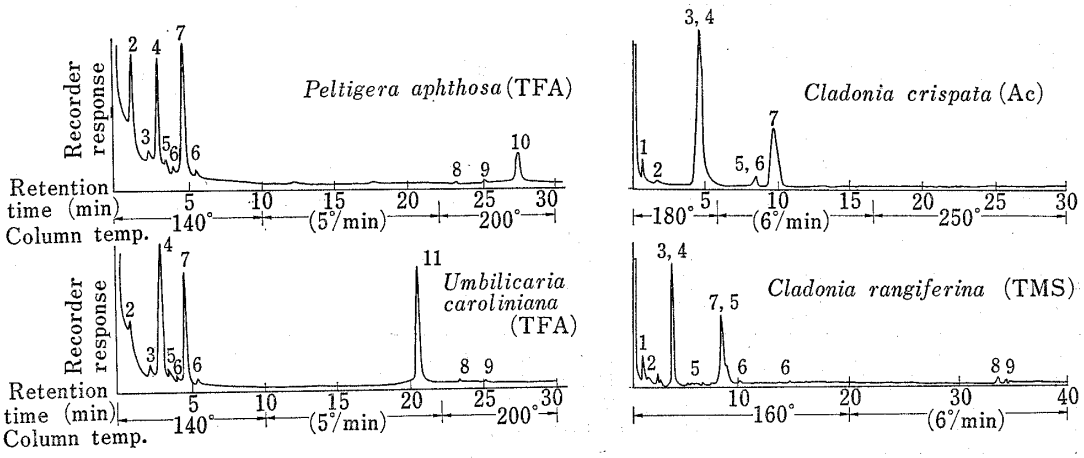

Fig. 2. Representative Gas Chromatograms

peak: 1: glycerol, 2: erythritol, 3: ribitol, 4: arabinitol, 5: fructose, 6: glucose, 7: mannitol, 8: sucrose, 9: trehalose, 10: mannitol-glucoside, 11: umbiiicin
Gas chromatographic conditions are described in the experimental part.

Umbilicin, 2-O-β-D-galactofuranosyl-D-arabinitol, was originally isolated from *U. pustulata*, and was subsequently discovered in *U. rigida*, *C. islandica*, and *Haematomma ventosum* by Lindberg, *et al.*[9,14] More recently, Holligan and Drew have investigated three *Umbilicaria* lichens, *U. polyphylla*, *U. pustulata*, and *U. torrefacta*, by means of gas chromatography. They found the presence of a compound presumed to be umbilicin only in the first named lichen, while of free galactose in the other two species.[7] Thus they suggested the alternative occurrence of the two constituents in these lichens. Our present finding that the two lichens, *U. caroliniana* and *C. islandica*, in which umbilicin was shown to be present, did not contain galactose has provided the additional examples to support their suggestion. In a

survey of ten species, Lindberg, *et al.* revealed that nine of them contained 3-O-β-D-glucopyranosyl-D-mannitol[7,11,15] as a sole glycoside constituent, while the tenth, *P. horizontalis* contained a large amount of 3-O-β-D-galactofuranosyl-D-mannitol (peltigeroside),[11b,15,16] along with a small amount of the former glycoside. It is, therefore, not surprising that only the mannitol-glucoside was detectable in two *Peltigera* lichens examined by us. Recently the occurrence of the glucoside has also been found in *Solorina crocea* (Peltigeraceae).[7]

Besides the carbohydrates mentioned above, the followings have been known to occur in some special lichens: arabinose, xylose, and a mannitol-mannoside (unknown structure) in *Lichina pygmaea*,[17] myo-inositol in *P. aphthosa*,[11a] volemitol in many species of the Pyrenocarpeae and also in *S. crocea*,[3,4,7,9] siphulitol (1-deoxy-D-glycero-D-talo-heptitol) in *Siphula ceratites*,[18] and a di- and a tri-saccharide consisting of tagatose and galactose in two *Roccella* lichens.[19] The absence of arabinose, xylose, galactose, myo-inositol, rhamnitol, sorbitol, and galactitol in the present lichens has been clearly demonstrated by direct gas chromatographic comparison with authentic samples. Other previously reported carbohydrates, though their authentic specimens were not available, seem to be virtually absent in the lichens examined, since the unidentified peaks (Table II, Fig. 2) were all found to be negligibly small.

For comparison, the present study involved three lichens whose carbohydrate constituents had been reported. The previously known carbohydrates of *C. islandica* are arabinitol, mannitol, sucrose, trehalose, and umbilicin.[9] Similar constituents except umbilicin had been found in *C. rangiferina*.[9,10] Our gas chromatographic analyses have now detected five additional carbohydrates, *e.g.*, glycerol, erythritol, ribitol, fructose, and glucose, in both the lichens. In the case of *P. aphthosa*, the situation is rather complicated. Lindberg's investigation by paper- and column-chromatography mentioned the presence of arabinitol, mannitol, myo-inositol, sucrose, trehalose, and the mannitol-glucoside in the lichen.[9,11] Subsequently, however, Holligan and Drew indicated that analyses by gas chromatography failed to detect sucrose, as well as myo-inositol, in any extracts of the several samples of this lichen, but did show small amounts of glycerol, ribitol, fructose, and glucose to be present.[7] Although our data of this lichen, both qualitative and quantitative, were found to be generally similar to their results, it should be mentioned that the specimen analysed by us contained sucrose, albeit in trace amounts, and also two so far unreported constituents, erythritol and trehalose, but did not contain myo-inositol.

Several factors may have contributed to such differences as observed in carbohydrate compositions of *P. aphthosa*. The variation may be due either to different habitats of the samples tested or to different procedures employed for analyses. Another possible explanation resides in seasonal variation in carbohydrate constituents, since a number of authors have previously indicated that the polyol contents vary with season remarkably.[3,16b,17] It is also important to note that we used the lichens which had been stored at room temperature for several years. Significant decline in carbohydrate contents must have occurred during the starvation. Therefore, it would be of special interest to compare the present data with those of freshly collected thalli in the future.

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16) *a*) G. Pueyo, *Rev. Bryol. Lichénol.*, **29**, 124 (1960); *b*) *Idem*, *Annéebiol.*, **36**, 117 (1960); *c*) N.K. Kochetkov, A.J. Khorlin, and A.F. Bochkov, *Tetrahedron*, **23**, 693 (1967).
17) G. de Lestang Laisné, *Rev. Bryol. Lichénol.*, **34**, 346 (1966).
18) B. Lindberg and H. Meier, *Acta Chem. Scand.*, **16**, 543 (1962).
19) B. Lindberg, *Acta Chem. Scand.*, **9**, 917 (1955).