[Chem. Pharm. Bull. 17(10)2061—2064(1969)]

UDC 581.19 : 581.13.08 : 547.98

Biosynthesis of Natural Products. VII.[1] Biosynthesis of Usnic Acid in Lichens. Seasonal Variation observed in Usnic Acid Biosynthesis

Heihachiro Taguchi,[2a] Ushio Sankawa and Shoji Shibata[2]

Faculty of Pharmaceutical Sciences, University of Tokyo[2]

(Received March 17, 1969)

The incorporation of sodium acetate (2-^{14}C) and $^{14}CO_2$ had been investigated in every two to four months to prove seasonal variation in usnic acid biosynthesis. In *Parmelia caperata* and *Usnea diffracta*, collected in different places, the highest incorporation was observed in February and the lowest in July. The alteration of experimental conditions gave no effect upon the results and it was concluded that the activity of usnic acid biosynthesis is effected by the seasonal condition.

Some seasonal variations have been observed in physiological activities of lichens. Wilhelmson[3] showed that the content of chlorophyll and the activity of photosynthesis are in a higher levelinw in ter than in any other seasons. Smith[4] reported that dry weight per unit area of lichen thallus, and the amount of glucose absorbed from the culture solution per day gave the highest value in late winter. The content of polyol in lichen was shown to give the highest value in spring or early summer and the lowest in winter.[5] On the other hand the growth rate of lichen is higher in summer than in winter.[6] A seasonal variation in the production of usnic acid was encountered during the course of the biosynthetical experiments, with which the present paper mainly concerns.

Materials and Methods

Labelled Compounds——Sodium acetate (2-^{14}C) and $BaCO_3$ (^{14}C) were purchased from Dai-ichi Chemical Co., Ltd. and methylphloroacetophenone (CO^{14}Me) was prepared as described in the previous paper.[1]

Feeding Experiments——The procedure of soaking method, dry feeding method and the method of CO_2 feeding were described in the previous paper.[1] All the feeding experiments were carried out at 25—27° unless otherwise stated.

Lichens——*Usnia diffracta* Wain. was collected at Lake District of Mt. Fuji, and *Parmelia caperata* Ach. was collected at Sugadaira, in Nagano-Ken.

Isolation of Usnic Acid and Diffractaic Acid from *U. diffracta*——The lichen thalli harvested after feeding experiment were washed thoroughly with water and dried by pressing between filter papers. The thalli were extracted with ether, and the solvent was removed from the extracts. The solid residue was treated with hot benzene, and the insoluble substance was separated by filtration. The benzene solution was charged on a silicic acid column made in benzene, and the column was developed with benzene and subsequently with chloroform. Usnic acid was obtained when solvent was removed from the benzene eluate. For the measurement of radioactivity, usnic acid was recrystallized repeatedly until a constant specific activity was attained. The compounds insoluble in benzene and the residue of the chloroform fraction obtained on evaporation of solvent were combined and recrystallized repeatedly to get a constant specific activity. Pure diffractaic acid melts at 196°.

1) Part VI: H. Taguchi, U. Sankawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 2054 (1969).
2) Location: *Hongo, Tokyo; a)* Present address: *Tsumura Institute, 1421, Izumi, Komae-machi, Kitatama-gun, Tokyo.*
3) J.B. Wilhelmson, *J.B. Botan. Tdsskr.*, **55**, 30 (1959).
4) D.C. Smith, *Lichenologist*, **1**, 209 (1961).
5) D.H. Lewis and D.C. Smith, *New Phytologist*, **66**, 143 (1967).
6) M.E. Hale, Jr., "The Biology of Lichen," Edward Arnold (Publisher) Ltd., London, 1967, pp. 81—82.

Isolation of Usnic Acid and Protocetraric Acid from *P. Caperata*——Lichen thalli washed with water and dried were extracted first with benzene and then with acetone. Usnic acid was isolated from benzene extracts, while protocetraric acid was obtained on evaporation of acetone from the extracts, which was recrystallized repeatedly from acetone. It showed mp>250°.

Measurement of Radioactivity——The same procedure was used as described in the previous paper.[1]

Results and Discussion

In the earlier stage of our investigation on the biosynthesis of usnic acid, a very low incorporation ratio from sodium acetate (2-^{14}C) into usnic acid was observed when *U. diffracta* was collected in June. In the same experiment diffractaic acid isolated along with usnic acid showed thirty times higher specific activity than that of usnic acid. However the feeding experiment using the lichen collected in February gave an entirely reverse result. The specific activity of usnic acid was thirty times higher than that of diffractaic acid. Moreover the incorporation ratio of ^{14}C into usnic acid was much higher, about 100 times, than that observed in June. The remarkable change of the incorportion ratio seemed to suggest a seasonal variation of biosynthesis of usnic acid. Prior to the study on the seasonal variotion, some investigation was made to clarify if there is any difference in the biosynthetical activity by the part of lichen thallus. A round thallus of *Parmelia caperata* was fed with $^{14}CO_2$ and divided into four parts, outer, outer middle, inner middle and inner. The specific acitvity of usnic acid and the activity per mg of thallus are listed in Table I.

Table I

Part	Usnic acid dpm/mM	Thallus dpm/mg
Outer	7.1×10^6	1.8×10^4
Outer middle	4.1×10^5	3.4×10^3
Inner middle	3.4×10^5	2.6×10^3
Inner	2.1×10^5	1.8×10^3

The results clearly showed that the outer part of the thallus is most active in secondary metabolism and photosynthesis. Since then all the feeding experiments were carried out by using small pieces of thalli which had been cut from whole lichen thalli and mixed thoroughly. The present results were recorded in every two to four months from May 1965 to August 1967 under the same experimental conditions. Sodium acetate (2-^{14}C) and $^{14}CO_2$

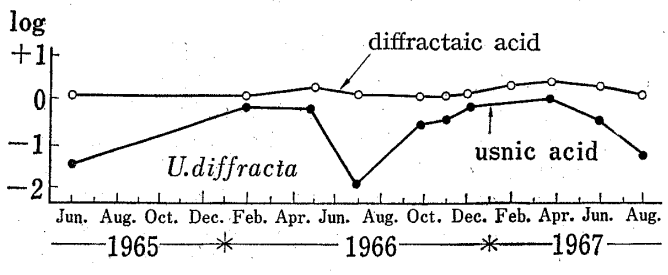


Fig. 1

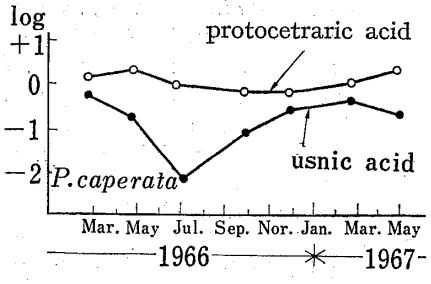
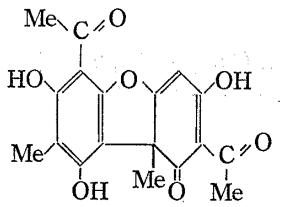
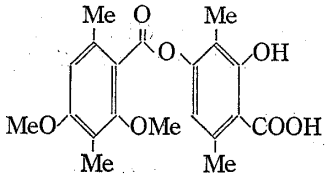


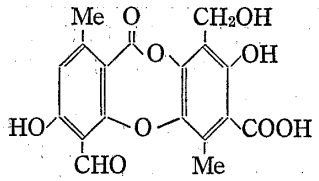
Fig. 2



usnic acid



diffractaic acid



protocetraric acid

were fed to *U. diffracta* and sodium acetate (2-^{14}C) only was fed to *P. caperata*. Three series of experiments were designed employing two different species, tracers and experimental conditions. The results are presented in Fig. 1 and 2.

The highest ^{14}C-incorportaion ratio into usnic acid was observed in February to April, and the lowest in July in every year. There is no remarkable difference between two different species of lichens as far as usnic acid concerns. The ^{14}C-incorporation ratios into diffractaic acid and protocetraric acid also showed a seasonal variation, but it is not so prominent as observed with usnic acid. In both cases the incorporation ratios are at the highest level in April or May. The experiment using $^{14}CO_2$ gave further support to the seasonal variation. The ratio of $\frac{\text{the specific activity of usnic acid}}{\text{the specific activity of diffractaic acid}}$, which was calculated from the data of the feeding experiments using sodium acetate (2-^{14}C) and $^{14}CO_2$, are shown in Fig. 3. In both cases, the variations are quite silmiar, and it is concluded that the variation is not caused by the different permeability of sodium acetate (^{14}C) to reach the active sites of the biosynthesis of these compounds.

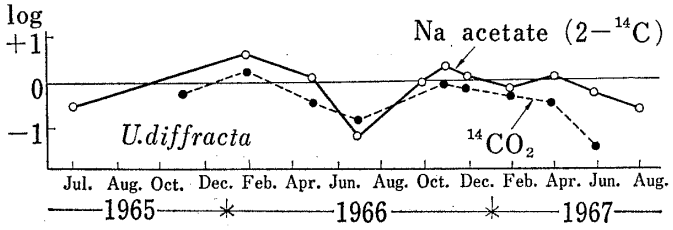


Fig. 3

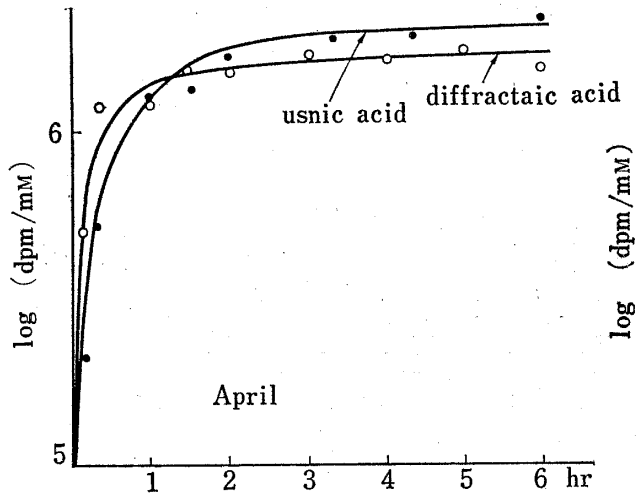


Fig. 4

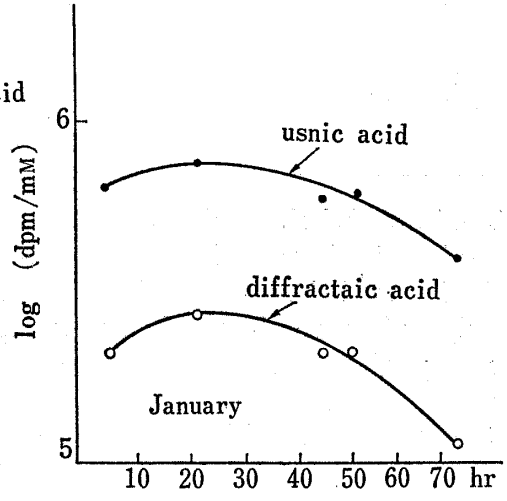


Fig. 5

The Fig. 4 and 5 show the change of the specific activity of usnic acid and diffractaic acid during the normal experimental period. Their specific activities increase unexpectedly fast during the first one hour and reach their maxima at about 20 hr after administration. The result excludes the possibility that the variation is caused by the rapid decomposition of usnic acid or by the quite different rate of incorporation into these two compounds. The change of the temperature in feeding experiment gave no significant effect upon the ratio. The data shown in Table II were obtained by feeding sodium acetate (2-^{14}C) to *P. caperata* at two different ranges of temperatures, 25—27° and room temperature (1—11°), with dry feeding method.

It is excluded from these results that the seasonal variation was caused by change of the natural environment of lichen, and the seasonal variation of ^{14}C incorporation is actually resulted by the change of the biosynthetical activity of lichen in natural state.

Table II

Temperature (C°)	Substance	Specific act. $\times 10^6$ dpm/mM	Ratio of specific activity
25—27	usnic acid	2.6	1.5
	protocetraric acid	1.7	
1—11	usnic acid	6.3	1.6
	protocetraric acid	4.0	

The further experiment was carried out to prove which step of biosynthesis of usnic acid is activated in winter. The course of biosynthesis of usnic acid could be divided into several steps, the formation of polyketide, its cyclization, phenolic oxidative coupling, the formation of the ether linkage and dehydration.[1)]

Table III

Labelled compound	Month	Amount added (μ ci)	Wt. of lichen	Amount added (μ ci)/Wt. of lichen	Usnic acid: Total act. (mμ ci)	Usnic acid: Spec. act. $\times 10^5$ dpm/mM	Inc. ratio %
Na acetate (2-^{14}C)	Aug.	10	5.5	1.8	2.4	0.31	0.034
	Aug.	4	2.5	1.6	2.1	0.33	0.055
	Dec.	20	10.0	2.0	61.2	2.2	0.31
	Dec.	20	9.0	2.2	82.0	3.8	0.41
Methyl phloroacetophenone (CO^{14}Me)	Aug.	1.5	5.5	0.27	47.3	4.8	3.5
	Aug.	1.5	3.5	0.31	36.1	4.8	2.7
	Dec.	0.5	4.0	0.12	39.2	3.6	7.5

The Table III shows the results of the experiments in which the ^{14}C-incorporation ratios into usnic acid from sodium acetate (2-^{14}C) and methyl phloroacetophenone (CO^{14}Me) were examined using lichens collected in December and August. Sodium acetate (2-^{14}C) gave ten times higher incorporation ratio by the experiment in December than that in August. However methyl phloroacetophenone (CO^{14}Me) showed only twice higher ratio by the experiment in December compared with that in August. It seems quite probable to conclude, therefore, that the early stage of biosynthesis of usnic acid is effected by the seasonal conditions.