Phytochemistry, 1976, Vol 15, pp. 1135–1138. Pergamon Press. Printed in England.

MANNITOL IN THE RHODOPHYCEAE—A REAPPRAISAL

BRUNO P. KREMER
Botanisches Institut der Universität, D-5000 Köln 41 (FRG)

(*Revised received* 9 *February* 1976)

Key Word Index—Rhodophyceae; red algae; mannitol; occurrence; metabolism; reinvestigation.

Abstract—Experiments were performed to determine whether mannitol occurs as a native constituent of marine and freshwater Rhodophyceae. Those red algae which had previously been reported to contain mannitol were tested. In none of these species could mannitol or any other hexitol be detected, either as ^{14}C-assimilate after photosynthetic assimilation of ^{14}C from $H^{14}CO_3^-$ or in trace amounts of the EtOH-soluble fraction. Attempts to qualify the action of a specific mannitol synthesizing enzyme (mannitol-1-phosphate dehydrogenase, EC 1.1.1.17) also failed. Though mannitol [^{14}C] is taken up when exogenously supplied by a seawater medium, less than 5% of ^{14}C from mannitol [^{14}C] taken up after 3 hr is recovered in other compounds. Mannitol is therefore not regarded as a natural metabolite (assimilate) of Rhodophyceae.

INTRODUCTION

The low-MW carbohydrates of the red algae have attracted very much attention in the past. Certain heterosides, e.g. 2-*O*-glycerol-α-D-galactopyranoside (floridoside), are characteristic constituents of this group [1,2] and there is a wide distribution of such galactosides in the Rhodophyceae [3,4].

Besides the well documented occurrence of glycerol galactosides there are occasional reports of the occurrence of the alditol mannitol in Rhodophyceae [5–7]. Though absolute amounts have been given in only a few cases [8–11], other data on mannitol such as rates of ^{14}C-labelling after photosynthetic $^{14}CO_2$-assimilation [12–18] also indicate physiologically relevant dimensions. In contrast to these reports, investigations of the typical ^{14}C-assimilate pattern have provided no evidence of involvement of mannitol [^{14}C] in the characteristic photosynthates of more than 60 red algal species [19]. Therefore the concept of mannitol as a systemic accumulation product or a widespread constituent of Rhodophyceae seems doubtful. On the other hand, mannitol may occur in measurable amounts, but is scarcely traceable in radiochromatographic analysis due to very low rates of ^{14}C-labelling like that of *iso*-floridosides [14]. Further attempts have now been made to isolate and trace mannitol from marine and freshwater Rhodophyceae.

RESULTS

Comparative radiochromatographic analyses

In the solvent systems often succesfully used for the TLC separation of plant metabolic products [20], the isomeric alditols are hardly distinguishable from each other, from their corresponding hexoses and pentoses, or even from some related carbohydrates such as glycerol galactosides.

In contrast to earlier results [20], it was found, for instance, that mannitol [^{14}C] from *Fucus serratus* and floridoside [^{14}C] from different species of Rhodophyceae show rather similar mobilities and are thus virtually indistinguishable after two-dimensional TLC (Fig. 1). However, when the EtOH-soluble fraction of any red alga (except the members of the Ceramiales, cf. ref. [19]) is separated by TLC according to [20] and the strongly ^{14}C-labelled compound which has been assumed to be mannitol [15–18] is re-eluted and subjected either to cautious acid hydrolysis or to treatment by α-galactosidase, two different spots are regularly observed after TLC in various solvent systems After such special treatment, the 'mannitol'-region of autoradiographs prepared from extracts of marine or freshwater Rhodophyceae after photosynthesis in $H^{14}CO_3^-$ exclusively yields a sugar identical with galactose [^{14}C] from *Elysia viridis* [21] and glycerol detectable by its characteristic colour reaction with the periodate/benzidine reagent. Furthermore, in the position of glycerol galactoside re-eluted from two dimensional TLC no mannitol [^{14}C] is implicated (Fig. 2).

Attempts to detect mannitol

Some authors reported the occurrence of considerable amounts of polyols in diverse Rhodophyceae. Table 1 summarizes recent reports of mannitol in red algae and shows the results of the reinvestigation of these and some further species. Mannitol is not detectable by the colour reaction of the spray reagent of Cifonelli and Smith [25]. The sensitivity of this method was found to be easily sufficient to demonstrate amounts of alditols less than 0.1% on a dry wt basis of the samples investigated. Mannitol was found in samples of *Dumontia incrassata* and *Rhodomela confervoides*; however, on microscopic examination these had proved to be considerably contaminated by epiphytic diatoms.

Enzyme assays

Provided that mannitol is really a widespread metabolite of Rhodophyceae it is expected that typical key

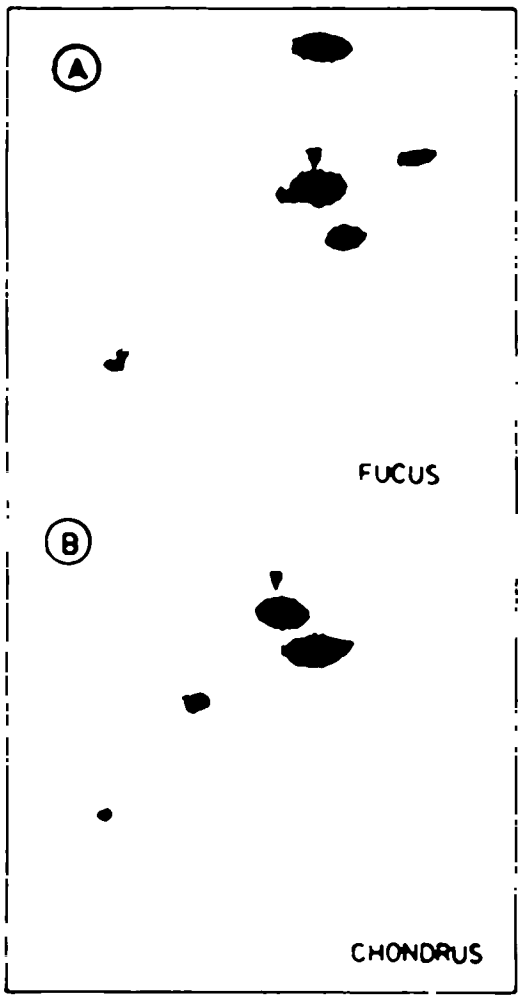

Fig. 1. Two dimensional TLC of EtOH-soluble fraction of (A) *Fucus serratus* and (B) *Chondrus crispus*: autoradiographs of ^{14}C-labelled assimilates. Spots indicated by arrow (mannitol and floridoside, resp.) are practically indistinguishable on the autoradiographs. For separation cf. ref. [20].

enzymes of mannitol metabolism, either mannitol dehydrogenase (EC 1.1.1.67) or mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) would be present in species of the red algae.

Enzyme preparations from *Giffordia mitchellae* and *Laminaria saccharina* (both Phaeophyceae) show high activities of mannitol-1-phosphate dehydrogenase as is indicated by proportionality and linearity of this enzyme reaction. No activity of mannitol-1-phosphate dehydrogenase, however, was found in enzyme extracts from the Rhodophycean species *Chrondrus crispus* or *Porphyra umbilicalis*. The same was true for *Ceramium rubrum* and *Polysiphonia urceolata*. Any further NADH-linked dehydrogenase reducing substrates other than fructose-6-phosphate to mannitol, such as glucose-6-phosphate, or even fructose, glucose, galactose, could not be found in the Rhodophyceae investigated.

Uptake and metabolism of exogenously supplied mannitol [^{14}C]. In all species investigated, mannitol [^{14}C] is taken up by the thallus samples from the seawater medium. The rate of uptake is of about the same order of magnitude in *Porphyra umbilicalis*, *Chondrus crispus*, and *Rhodomela confervoides*. In terms of absolute activity, the thallus samples did not remove more than 1% of the total amount of mannitol [^{14}C] supplied, even after incubation periods of at least 3 hr. This indicates rather slow rates of mannitol influx into the cells of the thallus of diverse species. The influx is not a linear function of incubation time, but shows typical saturation kinetics. Furthermore the alditol uptake is enhanced 2–3-fold under conditions of photosynthesis.

The data of the chromatographic analyses of ^{14}C-labelled compounds are shown in Table 2. The vast majority of radiocarbon recovered after mannitol uptake experiments is found in mannitol itself in all species.

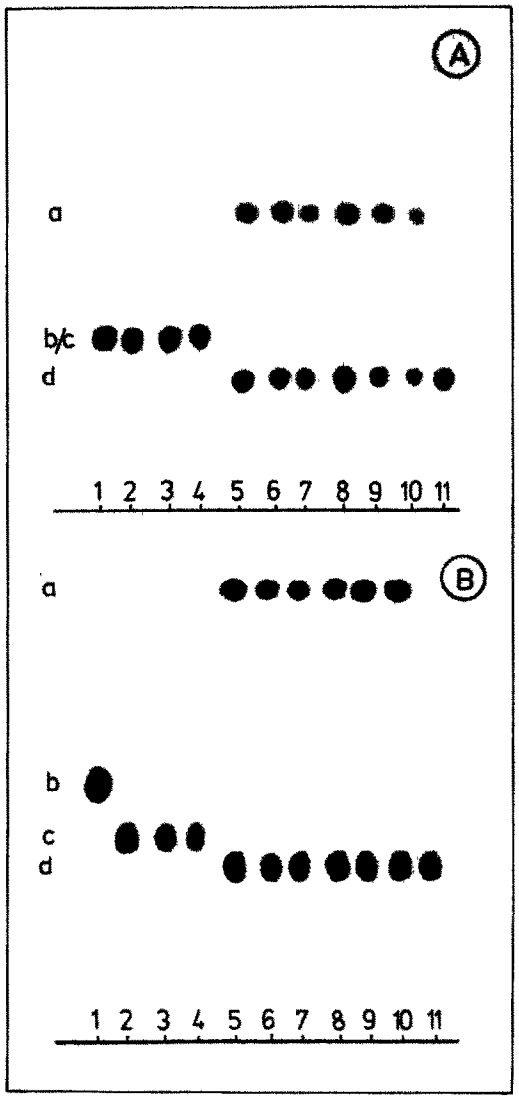

Fig. 2. Identity of mannitol [^{14}C] and floridoside [^{14}C] in one-dimensional TLC. Separation of (A) in solvent (1) of ref. [20], (B) in solvent (1) of ref. [31]. 1: mannitol [^{14}C] from *Fucus serratus*, 2,3,4: *floridoside* [^{14}C] from *Chondrus crispus*, *Dilsea carnosa*, *Batrachospermum* cf. *boryanum*; 5,6,7: floridoside of spots 2–4 hydrolized with 6 N HCl; 8,9,10: floridoside of spots 2–4 treated with α-galactosidase; 11: galactose ^{14}C from *Elysia viridis*. a: glycerol, b: mannitol, c: floridoside, d: galactose.

Table 1. Reinvestigation of some species with reported occurrence of mannitol

Species	Ref. to occurrence of mannitol	Reinvestigation and identification of Mannitol [^{12}C]	Mannitol [^{14}C]	Floridoside [^{14}C]
BANGIALES				
Porphyra umbilicalis	[32]	–	–	+
NEMALIONALES				
Batrachospermum cf. boryanum	[15	–	–	+
Lemanea fluviatilis	[15–17]	–	–	+
GIGARTINALES				
Ahnefiltia plicata	[13]	–	–	+
Chondurs crispus	[10, 14]	–	–	+
Cystoclonium purpureum	[13]	–	–	+
Furcellaria fastigiata	[22]	–	–	+
CRYPTONEMIALES				
Corallina officinalis	[13]	–	–	+
Dilsea carnosa	—	–	–	+
Polyides rotundus	[13]	–	–	+
RHODYMENIALES				
Halosaccion ramentaceum	[12]	–	–	†
Rhodymenia palmata	—	–	–	+
CERAMIALES				
Ceramium rubrum	[13]	–	–	–
Delesseria sanguinea	–	–	–	–
Membranoptera alata	–	–	–	–
Polysiphonia fastigiata	[24]	–	–	–
Polysiphonia nigrescens	[13]	–	–	–
Polysiphonia urceolata	–	–	–	–
Rhodomela larix	[9]	–	–	**
Rhodomela confervoides	–	–	–	–
Wrangelia penicellata	[11]	–	–	**

* cf. [19]. † not checked.

About 95% of total ^{14}C, on the average, is confined to this polyol. Only 1.2–6.3% of radiocarbon is distributed among the fraction of phosphorylated compounds and tricarboxylic acid cycle intermediates after incubation periods of to 3 hr, suggesting slow conversion of mannitol via respiration. These results indicate that mannitol uptake involves relatively slow metabolism of this alditol.

DISCUSSION

Comparative radiochromatographic studies in the pattern of ^{14}C-labelled photosynthates revealed that a compound assumed to be mannitol in a number of recent publications [15–18, 20] is really identical with 2-*O*-D-glycerol-α-D-galactopyranoside. In these cases, mainly concerning freshwater Rhodophyceae, the reports of mannitol as a common assimilate of red algae may simply be due to misidentification. Further attempts to trace mannitol in the EtOH-soluble fractions also failed. Mannitol was indeed found in thallus samples known to be associated with epiphytic diatoms; and even relatively small quantities of epiphytic or endophytic algae can cause severe alterations of the typical assimilate pattern [26]. The amounts of mannitol evaluated from red algal thalli may reflect the degree of such biological contamination [8,22,23]. Some uncertainty may also derive from a confusion of mannitol and mannose or mannose heterosides, as many Rhodophyceae, especially the members of the Ceramiales, contain mannoglycerate. Mannitol has convincingly been traced in *Holmsella pachyderma* [27], but this red alga lives parasitically and may thus not exhibit the normal metabolic situation.

The results are consistent with the data from some enzyme experiments. Tests of a key enzyme of mannitol biosynthesis (reducing direction) first characterized from brown algae [28] and confirmed in two further species, *Giffordia mitchellae* and *Laminaria saccharina*, revealed no activity of any mannitol dehydrogenases. Enzymes of this type would be expected if hexitols are typical constituents of Rhodophyceae.

Table 2. Distribution of ^{14}C (in %) recovered from some Rhodophyceae after long term uptake of mannitol [^{14}C] in the light (L) and in the dark (D)

Conditions	*Porphyra umbilicalis* Mannitol	*Porphyra umbilicalis* Others	*Chondrus crispus* Mannitol	*Chondrus crispus* Others	*Rhodomela confervoides* Mannitol	*Rhodomela confervoides* Others
1 hr L	97.4	2.6	98.0	2.0	98.8	1.2
3 hr L	96.2	3.8	97.3	3.7	98.2	1.8
1 hr D	93.8	6.2	96.2	3.8	96.9	3.1
3 hr D	93.7	6.3	96.0	4.0	96.1	3.9

Data from mannitol uptake experiments support the hypothesis that Rhodophyceae lack this alditol. Although mannitol is taken up actively and uptake is enhanced in the light, it is metabolized to a very low extent, and even this may be due to bacterial action which is not easily overcome when working with naturally grown material.

It was the purpose of the present study to determine whether mannitol indeed occurs as a general metabolite of marine and freshwater Rhodophyceae. As a result of the experiments presented here it may be reasonable not to regard mannitol as commonly occurring constituent of the Rhodophyceae. This qualification is consistent with the results of a recent study on the carbohydrate composition of various Rhodophyceae [29] which does not mention this or any other alditol.

EXPERIMENTAL

Giffordia mitchellae was taken from a laboratory culture. The other marine species were collected near Roscoff (Brittany, France) or near Helgoland (North Sea, Germany). Fresh water species were taken from various localities. Some further marine species were kindly supplied by colleagues as air-dried material (cf. acknowledgements).

^{14}C-incubation. Photosynthetic ^{14}C-assimilation from $H^{14}CO_3^-$ was established by incubation of carefully cleaned thallus samples (microscopically checked for diatoms and other epiphytes under the conditions detailed in ref. [19]. After ^{14}C-photosynthesis the thallus samples were briefly rinsed in H_2O and immediately fixed in small vols of hot EtOH.

Analytical. Thallus samples were extracted in 80% and 50% EtOH (final ratio *ca* 1:10, w/v) by grinding in a mortar. TLC of crude extracts was carried out according to ref. [20], prefractionated by ion exchange chromatography according to ref. [30]. The neutral fraction was further separated by TLC using the method of ref. [31]. Hydrolysis of floridoside [^{14}C] was performed either in 6 N HCl (20°, 10 hr) or by treatment with α-galactosidase (EC 3.2.1.22, Boehringer) in 0.1 M Pi-buffer pH 6.5. Alditols were detected by the periodate/benzidine reagent [25] (lower limit < 0.5 μg or < 0.1 μg after PC and TLC, resp.).

Enzyme assay. For the preparation of mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) *ca* 2 g thallus material (fr. wt) was homogenized with 10 ml 0.1 M Tris–HCl buffer pH 7.2, containing 20 μmol EDTA, 20 μmol D-araboascorbic acid, 20 μmol $MgCl_2$, 20 μmol dithiothreitol (DTT), 2 g Polyclar AT (prehydrated with 8 ml buffer) [32]. The resulting supernatant after centrifugation at 20000 *g* was used as enzyme extract. The reaction mixture contained 0.5 M Tris–HCl buffer pH 7 (1.6 ml), 1 μmol NADH (0.5 ml), 0.05–0.4 ml enzyme extract. The reaction was started with 50 μmol (0.5 ml) fructose-6-phosphate [28].

Polyol uptake experiments. Uptake of mannitol [^{14}C] in the light and dark by different Rhodophyceae was investigated by incubating thallus samples in a seawater medium containing 10 μmol mannitol [^{14}C] (Amersham-Buchler, CFA 238) in 50 ml membrane-filtered seawater (3.3×10^{-6} M soln). After incubation the samples were briefly rinsed, fixed in liquid N_2, lyophilized and further analysed as above.

Acknowledgements—I wish to thank Prof. Dr. J. Willenbrink, Cologne, for collecting *Lemanea fluviatilis* in Val d'Herens/VS/Switzerland. Many thanks are due to Dr. I. Munda, Ljubljana, Dr. N. Zavodnik, Rovinj, and Dr. J. A. West, Berkeley, for kindly providing me with *Halosaccion ramentaceum*, *Wrangelia penicelata*, and *Rhodomela larix*, respectively. I wish to thank, too, Dr. James W. Markham for kindly correcting the English version of this manuscript.

REFERENCES

1. Colin, H. and Guéguen, E. (1930) *C.R. Acad. Sci. Paris* **191**, 163.
2. Putman, E. W. and Hassid, W. Z. (1954) *J. Am. Chem. Soc.* **76**, 2221.
3. Augier, J. (1947) *C.R. Acad. Sci. Paris* **224**, 1654.
4. Augier, J. and Du Mérac, M. L. (1954) *C. R. Acad. Sci. Paris* **238**, 387.
5. Meeuse, B. J. D. (1962) Storage Products, in *Physiology and Biochemistry of Algae* (Lewin, R. A., ed.) Academic Press, New York.
6. Lewis, D. H. and Smith, D. C. (1967) *New Physiol.* **66**, 185.
7. Craigie, J. S. (1974) *Storage Products*, in *Algal Physiology and Biochemistry* (Stewart, W. D. P., ed.), Blackwell, Oxford.
8. Nagashima, H., Nakamuras, S. and Nisizawa, K. (1969) *Bot. Mag.* **82**, 379.
9. Whyte, J. N. C. and Southcott, B. A. (1970) *Phytochemistry* **9**, 1159.
10. Buggeln, R. G. and Craigie, J. S. (1973) *Proc. N.S. Inst. Sci.* **27**, 81.
11. Zavodnik, N. (1973) *Bot. Mar.* **16**, 166.
12. Bidwell, R. G. S. (1958) *Can. J. Botany* **36**, 337.
13. Majak, W., Craigie, J. S. and McLachlan, J. (1966) *Can. J. Botany* **44**, 541.
14. Craigie, J. S., McLachlan, J. and Tocher, R. D. (1968) *Can. J. Botany* **46**, 605.
15. Feige, B. (1970) *Z. Pflanzenphysiol.* **63**, 288.
16. Feige, G. B. (1973) *Z. Pflanzenphysiol.* **69**, 290.
17. Feige, G. B. (1974) *Z. Pflanzenphysiol.* **72**, 272.
18. Feige, G. B. (1975) *Z. Pflanzenphysiol.* **75**, 339.
19. Kremer, B. P. and Vogl, R. (1975) *Phytochemistry* **14**, 1309.
20. Feige, B., Gimmler, H., Jeschke, W. D. and Simonis, W. (1969) *J. Chromatog.* **41**, 80.
21. Kremer, B. P. (1976) *Z. Pflanzenphysiol.* **77**, 139.
22. Lindberg, B. (1955) *Acta Chem. Scand.* **9**, 1093.
23. Lindberg, B. (1955) *Acta Chem. Scand.* **9**, 1097.
24. Wickberg, B. (1957) *Acta Chem. Scand.* **11**, 506.
25. Cifonelli, A. and Smith, F. (1954) *Anal. Chem.* **26**, 1132.
26. Kremer, B. P. (1975) *Helgol. Wiss. Meeresunters.* **27**, 115.
27. Evans, L. V., Callow, J. A. and Callow, M. E. (1973) *New Phytol.* **72**, 393.
28. Ikawa, T., Watanabe, T. and Nisizawa, K. (1972) *Plant Cell Physiol.* **13**, 1017.
29. Impellizzeri, G., Mangiafico, S., Orinete, G., Piatelli, M., Sciuto, S., Fattorusso, E., Magno, S., Santacroce, C. and Sica, D. (1975) *Phytochemistry* **14**, 1549.
30. Splittstoesser, W. E. (1969) *Plant Cell Physiol.* **10**, 87.
31. Kremer, B. P. (1975) *J. Chromatog.* **110**, 171.
32. Weidner, M. and Küppers, U. (1973) *Planta* **114**, 365.