Phytochemistry, Vol. 24, No. 1, pp. 43–45, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

SORBITOL, A PRECURSOR OF L-GULURONIC ACID IN ALGINIC ACID BIOSYNTHESIS

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(*Received* 8 *May* 1984)

Key Word Index—*Sargassum muticum*; Fucales; sorbitol; mannitol; guluronic acid; alginic acid; epimerase; biosynthesis.

Abstract—Radioactive D-[U-^{14}C]sorbitol 6-phosphate injected into young growing *Sargassum muticum* tips is transformed within a few hours into radioactive L-guluronic acid. A C_5-epimerase intervenes reversibly to balance the ratio of mannuronic and guluronic acids in the newly synthesized alginic acid.

INTRODUCTION

The alginic acid of brown seaweed cell-wall consists of a long succession of alternating homopolymeric units: poly-D-mannuronic units (MM) and poly-L-guluronic units (GG) separated by hybrid (MG) units of poly-(D-mannuronic-L-guluronic). The biosynthesis of algal mannuronic acid, starting from D-mannitol, using GTP and NAD coenzymes, has been clearly demonstrated. That of guluronic acid raises a more difficult problem which we think has not been satisfactorily resolved. Larsen and Haug [1, 2] suggest that the polyguluronic (GG) and hybrid polymer (MG) units arise from the polymannuronic units, by the action of a C_5-epimerase which they found in *Azotobacter vinelandii*, a bacterium producing an acetylated alginate. *In vitro*, this enzyme is in fact able to convert the purified MM into GG units, in the presence of calcium; Madgwick *et al.* [3] also detected a similar epimerase in the brown seaweed *Pelvetia canaliculata*. Nevertheless, it is an unusual pathway of biosynthesis, as the authors themselves remark [4]. Some objections arise: one wonders why a GDP-guluronic acid is found in seaweeds [5] if an epimerase is sufficient to produce the GG from the MM units; besides, the energy required for such an epimerization, which could come only from complexing with Ca^{2+}, seems to be insufficient.

A biosynthesis following the pathway of D-sorbitol, a polyol present in all the brown seaweeds studied [6], is much more probable, D-sorbitol being in effect identical to L-gulitol. L-Guluronic acid could result from the transformation of this L-gulitol into L-guluronic acid, by a process similar to the one which converts mannitol into mannuronic acid, using the same GTP and NAD coenzymes. So, the objections mentioned above would disappear. The present paper aims to demonstrate the above hypothesis (Scheme 1).

RESULTS AND DISCUSSION

Sargassum muticum, a common seaweed capable of rapid growth, was chosen as experimental material. Two series of experiments were carried out in 1982 and 1983, differing in the duration of assimilation of radioactive sorbitol, the season of harvesting, and the choice of vegetative tips.

D-[U-^{14}C]Sorbitol 6-phosphate free of mannitol and mannitol phosphate was injected into the tips of very young thalli in full growth. The alginate constituents were separated and analysed after several hours of assimilation. The specific radioactivity in each type of unit was measured, as well as that of the M and G components of the MG units (Table 1).

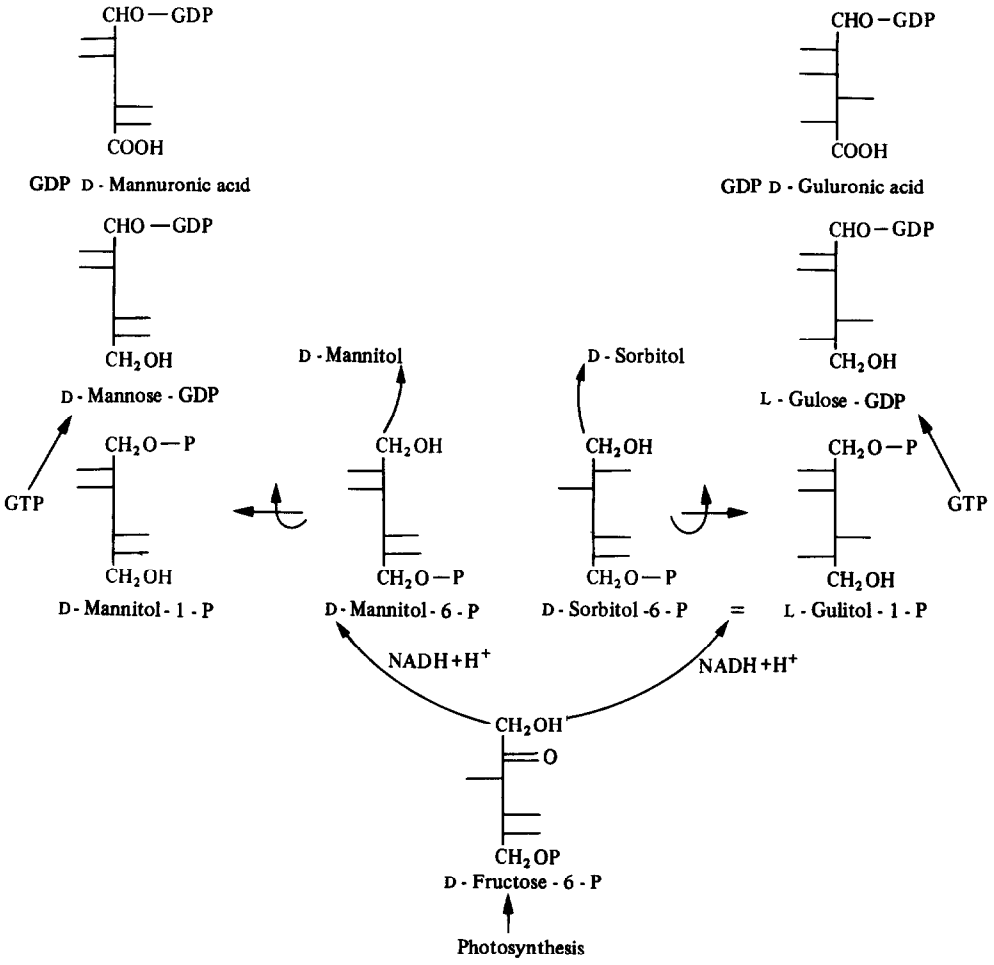
As observed by Haug, and as we have verified too, the MM and MG units are not entirely separated by the usual technique [7]; MM units contain 15–20% of MG units and vice versa. Taking into account the corresponding correction, it can be stated that after 24 hr the MM units are not radioactive whereas the GG blocks are clearly labelled; the MG units are much more radioactive, but on their G component only.

After 72 hr, MM units are not yet labelled. But the M component of the MG units is now radioactive, much more than would be expected from an incomplete experimental separation. It must be concluded that part of the synthesized guluronic acid is converted into this radioactive mannuronic acid, very probably by the action of the C_5-epimerase operating here from G to M, showing the reversibility suspected by Madgwick. The isomerization in the sense G to M requires ATP, normally furnished by the living organism. This energy was obviously lacking in the *in vitro* experiments of Larsen and Haug, who could only perceive the action from M to G.

Our results leave no doubt as to the reality of synthesis of L-guluronic acid from D-sorbitol, nor of the regulating activity of the C_5-epimerase discovered by Haug and Larsen. This biosynthetic pathway found in brown seaweeds, which we detected in *Fucus vesiculosus* as well, could well be absent in bacteria such as *Azotobacter vinelandii* since, to our knowledge, sorbitol has never been identified in such organisms.

EXPERIMENTAL

Preparation of the sorbitol 6-phosphate free of mannitol. D-[U ^{14}C]glucose 6-phosphate (Amersham; 9.25 MBq) was



Scheme 1.

Table 1. Activity of alginate components (dpm for 100 mg lactone)

	GG		MM		MG	
Assimilation time (hr)	Measured ($\times 10^3$)	Corrected (-16% MM) ($\times 10^3$)	Measured ($\times 10^3$)	Corrected (-16% GG)	Measured ($\times 10^5$)	Measured ($\times 10^5$)
24	72	90	15	0	10	G 20 M 0.1
72	350	420	70	0	40	G 39 M 34

evapd to 0.2 ml, then reduced by $NaBH_4$ (50 mg) during 2 hr at 20°. The borohydride was decomposed by 1 g strong resin (H^+) (Amberlite IR 120). Boric acid was eliminated from the filtrate as the methyl ester, by adding 2 ml MeOH and evapn under red. pres. (× 15). Final volume: 50 μl. This product, which could contain some residual glucose 6-phosphate or traces of mannitol phosphate arising from a possible slight epimerization in the alkaline medium, was purified by HP TLC (solvent: BuOH–pyridine–H_2O, 2:2:1) The plate was tested for P and polyol content, eluting tiny consecutive areas (10 mm^2) along a vertical band, as previously described [6], and tested for radioactivity by liquid scintillation. Two bands were well separated: the fastest one, of low activity and possibly containing some mannitol phosphate, was not eluted. The slowest (R_f 0.22) and main band, strongly labelled, contained D-[U-^{14}C]sorbitol 6-phosphate. It was eluted and evapd to 0.2 ml.

Inoculation. This radioactive, highly purified soln of D-sorbitol 6-phosphate was inoculated, using a micromanipulator, by numerous injections into the stipes and vesicles of the growing tips (5–15 cm long) of young *Sargassum muticum*. These pieces of

thalli were maintained, without washing, immersed in filtered seawater for 24 or 72 hr. They were then quickly blotted and put into boiling EtOH for 5 min. Dried and powdered, they were extracted in a BBS apparatus by boiling EtOH for 3 hr, then by 85% EtOH. Finally, the powder was washed with 50 ml HCl (pH 3) in order to discard fucoidan, laminarin, polyols, some proteins and possible traces of the marker. The alginate of the insoluble residue filtered on sintered glass was then extracted and the GG, MM and MG units were separated following the techniques of Haug *et al.* [7]; these pure fractions were dissolved in NaOH—an aliquot was used to evaluate the uronic acid content (phenol method); another fraction was introduced in a plastic vial, mixed with 7.5 ml of the ACS scintillation solution (Amersham) and the activity counted in a Beckman LS 7000 scintillator.

The MG units were fully hydrolysed using 72% H_2SO_4 [8], neutralized, and submitted to electrophoresis in a borate–$CaCl_2$ buffer [9]. The strips containing the separated uronic acids were eluted. One part was used to evaluate the uronic content (with borated carbazol) [10] and another to measure the radioactivity.

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