BETULAPRENOL PHOSPHATE AS AN ACCEPTOR OF MANNOSE FROM GDP-MANNOSE IN *PHASEOLUS AUREUS* PREPARATIONS

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1. Introduction

It is well established that undecaprenol phosphate may act as an acceptor of sugar residues from nucleoside diphosphate sugars in bacterial systems. The product, undecaprenol phosphate sugar or undecaprenol diphosphate sugar may then pass the sugar residue on to a polymer in the bacterial wall [1]. Since many higher plants have been shown to contain polyprenols [2, 3] the possibility of analogous lipid intermediates functioning in the biosynthesis of structural polysaccharides in plant cell walls has been considered. Initially, evidence of an acid-labile lipid intermediate in the transfer of mannose from GDP-mannose to polysaccharide by a particulate enzyme preparation of *Phaseolus aureus* was reported [4]. A more recent report [5] indicates the chromatographic similarity of this lipid to the undecaprenol phosphate mannose of *Micrococcus lysodeikticus* [6]. The incorporation of ^{3}H from 5-^{3}H-mevalonic acid into the lipid part of the mannolipid has been observed [7] and the possibility that a prenol phosphate is involved was strengthened by the discovery that betulaprenol phosphate and dolichol phosphate stimulate the incorporation of mannose from GDP-mannose into the lipid in a relatively specific way [8]. However, while all of these observations are consistent with prenol phosphates acting as mannose acceptors, they did not constitute proof of this. It seemed important to establish this point before other aspects of the relationship could be considered further. So far insufficient lipid 'intermediate' of adequate purity has been obtained for its characterization. Also the demonstration of the incorporation of prenol phosphate into the lipid by the usual radioisotopic methods has required a radioactive prenol phosphate of specific activity higher than has hitherto been available. This latter problem has now been largely overcome and in this paper the synthesis of ^{3}H-betulaprenol phosphate of relatively high specific activity is reported. It is also shown that a particulate preparation of seedlings of *Phaseolus aureus* will use this as an exogenous acceptor of ^{14}C-mannose from GDP-^{14}C-mannose, resulting in the formation of an acid-labile lipid that is chromatographically very similar to undecaprenol phosphate mannose.

2. Materials and methods

A betulaprenol preparation (50 mg) [3] was dissolved in light petroleum (b.p. 40–60°, 5 ml) and was oxidised to betulaprenal by refluxing for 2 hr with manganese dioxide (100 mg) [9]. The resultant mixture was poured onto a column of alumina (Brockmann Grade 3, acid-washed) which was then washed successively with mixtures of diethyl ether/light petroleum in the order 1/99, 1/49, 3/97 and 1/4. Betulaprenal (30 mg) was eluted by the first two eluents and betulaprenol by the last eluent. ^{3}H-Betulaprenal was then prepared by an exchange reaction. Betulaprenal (20 mg) was dissolved in dioxane (1 ml) and mixed with ^{3}H-water (1 ml, 1 mCi). A small pellet of KOH was added and the mixture was refluxed overnight. The ^{3}H-betulaprenal was recovered by extraction with ether and was reduced to ^{3}H-betulaprenol with excess sodium borohydride in methanol. This was purified by chromatography on alumina as above and by preparative thin-layer chromatography (TLC) on silica gel G using methanol/benzene 1/99 as solvent. The final product had a specific activity of 4.5 μCi/mg.

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The phosphate of ^{3}H-betulaprenol was prepared chemically and purified by chromatography on DEAE-cellulose acetate [8].

The ^{3}H-betulaprenol phosphate was incubated with a particulate fraction derived from 4-day old seedlings (approx. 20 g) of *Phaseolus aureus* [10]. This fraction was resuspended in 0.5 ml of medium containing phosphate buffer (0.05 M, pH 7.3), sucrose (0.4 M), $MgCl_2$ (0.01 M) and albumin (1%) and to this was added ^{3}H-betulaprenol phosphate (5×10^{5} dpm) suspended in a further 0.5 ml of medium with the aid of Triton X-100 followed by GDP-^{14}C-mannose (0.1 μCi, 70 nmoles). This mixture was incubated for 5 min at 27°. The lipid was extracted from the mixture using butanol (2 ml) followed by chloroform/methanol (2/1, v/v, 5 ml). The combined extracts were washed thoroughly with water and a portion of the lipid extracted was then chromatographed preparatively on thin layers of silica gel G using chloroform/methanol/water (65/25/4, v/v-system A) as solvent in order to separate the mannolipid formed from excess betulaprenol phosphate and from decomposition products. A broad band (Rf 0.3–0.45) of the chromatogram containing the mannolipid (usual Rf 0.35) was removed and extracted with chloroform/methanol (2/1, v/v saturated with water). A portion of this was then chromatographed on silica gel G using as solvent di-isobutyl ketone/acetic acid/water (60/45/6, v/v – system B). Successive bands of the chromatogram were then removed and assayed for ^{3}H and ^{14}C by liquid scintillation counting taking great care to correct accurately for quenching.

3. Results and discussion

The specific activity of the betulaprenol (4.5 μCi/mg) formed was not as high as expected but proved sufficiently high for the subsequent experiment. Appropriate modification of the exchange conditions would probably improve the specific activity. Mass spectrometry on the product of a parallel exchange reaction using deuterated water instead of tritiated water indicated that up to six atoms of hydrogen had been replaced by heavy isotope–almost certainly on the carbon atoms β, δ and δ' (CH_3 group) to the hydroxyl group.

Incubation of GDP-^{14}C-mannose with the enzyme preparation, in the presence of ^{3}H-betulaprenol phosphate led to the formation of ^{14}C-mannolipid (23,000 dpm). Radioassay of the mannolipid recovered after preparative chromatography in system A indicated that 1.3% of the ^{3}H added as ^{3}H-betulaprenol phosphate had become associated with ^{14}C-mannose. In this system betulaprenol phosphate has an R_f value of approximately 0.08 and phosphate-free decomposition products of betulaprenol phosphate run close to the solvent front and both are thus readily separated from the mannolipid. Most of the ^{3}H remained associated with ^{14}C when a portion of the purified mannolipid was rechromatographed in system B (fig. 1). In this system betulaprenol phosphate has an R_f of 0.62 and phosphate-free decomposition products of betulaprenol phosphate run close to the front. The recovery of some ^{3}H near the solvent front reflects the instability of the mannolipid and its partial decomposition during preparative chromatography in system A. Decomposition would be expected to yield polar ^{14}C-labelled compounds that would not be extracted from the chromatogram with chloroform/methanol and which would not appear, therefore, in fig. 1. Difficulties of preparative TLC of these mannolipids have been reported by other workers.

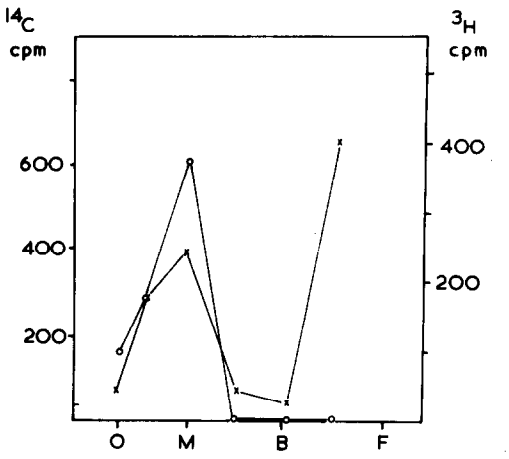

Fig. 1. The distribution of ^{14}C (o——o) and ^{3}H (x——x) along the chromatogram after chromatography, using system B, of a portion of the mannolipid recovered from chromatography in system A (see text). Each point represents the radioactivity recovered in a band 1 cm either side of the point. O = origin, F = solvent front, M = position expected for mannolipid, B = position expected for betulaprenol phosphate.

The relatively low incorporation was not unexpected considering the difficulty of bringing a lipid, not soluble in water, into correct contact with a particulate enzyme, and also considering the lability of the compound.

This experiment establishes the incorporation of ^{3}H of exogenous ^{3}H-betulaprenol phosphate into a lipid that has the same chromatographic properties as the ^{14}C-mannolipid in two systems capable of separating mannolipid from betulaprenol phosphate and from decomposition products. Coupled with the fact that this mannolipid resembles undecaprenol phosphate mannose in chromatographic properties, acid lability and alkali stability [11], and bearing in mind the results summarised in the Introduction, this is interpreted as strong evidence that exogenous betulaprenol phosphate can act as an acceptor of mannose from GDP-mannose in particulate fractions of *Phaseolus aureus*. This situation strengthens considerably the idea that prenol phosphates may function in plants in a manner analogous to that established in bacteria.

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