AN ARTIFICIAL MANNOSYL ACCEPTOR FOR GDP-D-MANNOSE: LIPID PHOSPHATE TRANSMANNOSYLASE FROM *PHASEOLUS AUREUS*†

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1. Introduction

Glycophospholipids are intermediates in the biosynthesis of bacterial cell wall components [1]. These lipids have been identified as sugar derivatives of isoprenol pyrophosphates [2, 3], or isoprenol monophosphates [4]. Similar compounds have been suggested to be intermediates in the synthesis of plant cell wall components [5–7]. The properties of a mannolipid synthesized in *Phaseolus aureus* enzyme preparations [6, 7], indicate a structural similarity to one identified as undecaprenol phosphate mannose [4]. However, quantities of this plant mannolipid sufficient for conclusive identification of the hydrocarbon moiety have been unavailable. This report describes a commercially available isoprenol, which appears to substitute for the endogenous mannosyl acceptor in *Phaseolus aureus* enzyme preparations. This provides a ready assay which can be used in the isolation and purification of the GDP-D-mannose:lipid phosphate transmannosylase from *Phaseolus aureus* hypocotyls, and provides data supporting earlier [5–7] suggestions concerning the probable nature of the lipid phosphate acceptor.

2. Experimental

The particulate enzyme was prepared from *Phaseolus aureus* hypocotyls in the manner described previously [5]. Reactions were performed by combining 50 μl particulate enzyme (about 0.5 mg plant protein), 40,000 cpm (about 60,000 dpm) GDP-α-D-[^{14}C]mannose (ICN, Irvine, California; specific activity 52 or 72 mCi/mM), plus any other additions in a final volume of 0.1 ml. Reaction times and temperature, as well as other reactants, are indicated in the figures. The reactions were terminated by the addition of 50 μl of 15% trichloroacetic acid.

Phytol (Nutritional Biochemical Company) was catalytically reduced to 3, 7, 11, 15-tetramethyl hexadecanol (Phytanol) using Pt in an ethanol solution under 10 psi H_2 pressure. The product was separated from the catalyst by centrifugation. Complete reduction was demonstrated by NMR spectroscopy and permanganate oxidation. Both phytanol and phytol were phosphorylated, using trichloroactonitrile as the condensing agent, by a modification of the method described by Popjak et al. [8–10]. Attempts to crystallize the derivatives were unsuccessful. Adjustment of the water solution to pH 2, then extraction with $CHCl_3$:MeOH, 1:1, resulted in good yields of a mixture of the mono- and pyrophosphate esters. The phytanol phosphate derivatives were subjected to a mild acid hydrolysis (0.1 N HCl, 100°, 6–7 min) yielding phytanol monophosphate with only traces of phytanol [11]. The 3, 7, 11, 15-tetramethyl hexadecanyl phosphate (phytanol phosphate) was extracted into $CHCl_3$:MeOH, evaporated to dryness, and washed with water, ether, and hexane to remove impurities. Thin-layer chromatography on silica gel H using chloroform:methanol, 1:1, gave only one lipid spot with rhodamine 6-G indicator and a coinciding single spot with Haines and Isherwood's spray for phosphate. The samples (0.01 moles/ℓ) were

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Table 1
Stimulation of [^{14}C]mannolipid formation.

Addition	[^{14}C]Mannolipid (% of Control Reaction)
None	100
Lauryl sulfate	92
Myristyl sulfate	101
Phytol	108
Phytanol	88
Phytol phosphate	162
Phytanol phosphate	570

Reactions and analyses were performed as described in the text. The additions were made at 0° just prior to initiation of the reaction. The reactions took place for 10 min at 40°. All additions resulted in a final concentration of 0.001 M additive.

suspended in water by sonication for 2 min at 90 W output with a Biosonic III sonic oscillator.

3. Results and discussion

Phosphate derivatives of phytol and phytanol stimulate the incorporation of radioactivity from GDP-α-D-[^{14}C]mannose into mannolipids (table 1). Neither phytol, phytanol, nor hydrocarbon sulfate derivatives stimulate the production of [^{14}C]mannolipid. Of the two stimulatory isoprenol phosphates, the saturated derivative phytanol phosphate, produced the greater stimulation.

The stimulatory effect of phytanol phosphate results from the production of an additional [^{14}C]mannolipid (fig. 1). The quantity of endogenous [^{14}C]mannolipid eventually formed is not affected by additions of phytanol phosphate, but the initial rate is slightly reduced. Kinetic studies demonstrate that the initial rate of new [^{14}C]mannolipid production is much slower (about 1/5 the rate at 40°) than that of endogenous [^{14}C]mannolipid production. However, endogenous [^{14}C]mannolipid production reached a steady state in a very short time (considerably less than 1 min at 40°), while the new [^{14}C]mannolipid quantity continues to increase for several minutes before reaching a steady state. As a result, at early time periods the additional [^{14}C]mannolipid represents only a small proportion of total

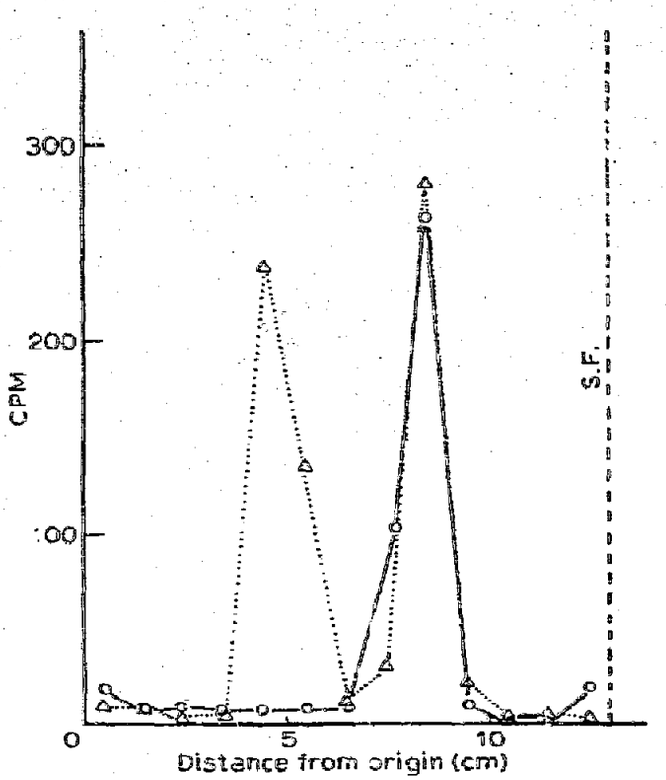

Fig. 1. Separation of [^{14}C]mannolipids formed in a control reaction and in a reaction containing 1 mM phytanol phosphate. Reactions and analyses were performed as described in the text. The reactions took place at 40° for 10 min. Thin-layer chromatography was performed using silica gel H and 12:6:1 $CHCl_3$:CH_3OH:H_2O as a solvent. Localization was by scraping off 1 cm sections and determining radioactivity by scintillation counting. Control reaction (o—o—o), and reaction containing phytanol phosphate (Δ—Δ—Δ).

[^{14}C]mannolipid, whereas after longer reaction times it represents a substantial proportion. For example, at 40° in 15 sec the new [^{14}C]mannolipid made up 15% of the total [^{14}C]mannolipid produced, but after 30 sec made up 34%, after 90 sec 53%, and after 10 min 89% of the total [^{14}C]mannolipid produced.

The enhancement of [^{14}C]mannolipid production increased with increasing concentrations of phytanol phosphate up to 0.01 M, the highest practical concentrations we could use. It was not possible to saturate the enzyme system within this concentration limit. Also, the stimulation of [^{14}C]mannolipid production (upon addition of phytanol phosphate) increased as a function of temperature varying from a stimulation of about 1.2 at 0° to a stimulation of about 6.5 at 40°. Above approx. 50° rapid enzyme inactivation was the predominant effect, with or without

Table 2
The effect of phytanol phosphate on [^{14}C]glycolipid synthesis from different substrates.

Substrate	Stimulation or inhibition (+ phytanol phosphate/control reaction)
GDP-[^{14}C]mannose	1.75
UDP-[^{14}C]glucose	0.89
UDP-[^{14}C]galactose	1.04
UDP-[^{14}C]glucuronic acid	0.65
UDP-[^{14}C]galacturonic acid	0.67
GDP-[^{14}C]glucose	No [^{14}C]glycolipid with or without phytanol phosphate
UDP-[^{14}C]xylose	No [^{14}C]glycolipid with or without phytanol phosphate

Reactions and analyses were performed as described in the test. The reactions took place for 10 min at 30°. The quantities of radioactive substrates used were: GDP-[^{14}C]mannose, 32,000 cpm, specific activity 55 mCi/mM; UDP-[^{14}C]glucose, 48,000 cpm, specific activity 233 mCi/mM; GDP-[^{14}C]glucose, 40,000 cpm, specific activity 162 mCi/mM; UDP-[^{14}C]galactose, 41,000 cpm, specific activity 200 mCi/mM; UDP-[^{14}C]galacturonic acid, 165,000 cpm, specific activity 127 mCi/mM; UDP-[^{14}C]glucuronic acid, 33,000 cpm, specific activity 100 mCi/mM; UDP-[^{14}C]xylose, 12,400 cpm, specific activity 150 mCi/mM. Final concentration of phytanol phosphate was 0.001 M.

phytanol phosphate. These results suggest that a permeation barrier is associated with the rate limiting step.

The chemical properties of the new [^{14}C]mannolipid are similar to those of endogenous [^{14}C]mannolipid (see [6, 7]). It is quantitatively bound by DEAE-cellulose indicating that it possesses a negative charge. The compound is acid labile: greater than 90% hydrolysis resulted from treatment with 0.1 M HCl for 10 min at 100°. Greater than 75% hydrolysis resulted from treatment with 0.01 M HCl for 10 min at 100°. From either acid hydrolysis, the only radioactive product was [^{14}C]mannose. The compound is relatively staule to base hydrolysis: using the conditions of Scher and Lennarz [4], no hydrolysis resulted, and less than 25% was hydrolyzed using the conditions of Wright et al. [3]. The latter conditions result in quantitative hydrolysis of isoprenol pyrophosphate saccharides. Also, the reaction forming the new [^{14}C]mannolipid is quantitatively reversed by 0.001 M GDP, but is not reversed by 0.001 M GMP.

Phytanol phosphate did not enhance the production of [^{14}C]glycolipid using any of the known sugar nucleotides tested (table 2) other than GDP-D-[^{14}C]mannose. However, an unknown compound would serve as a glycosyl donor. This radioactive compound was received as a purported shipment of GDP-D-[^{14}C]mannose. It was tentatively identified as GDP-[^{14}C]rhamnose on the basis of paper chromatographic mobility as the sugar nucleotide, and mobility of the [^{14}C]product of mild acid hydrolysis. The above results indicate that the enzyme catalyzing the formation of the [^{14}C]glycolipid in the presence of phytanol phosphate is relatively specific for GDP-D-mannose as a substrate.

The results indicate that phytanol phosphate serves as a substrate for the GDP-mannose:lipid phosphate transmannosylase found in *Phaseolus aureus* seedlings. While phytanol phosphate is a poorer glycosyl acceptor than the endogenous lipid phosphate substrate, it is readily available in gram quantities. The endogenous lipid phosphate acceptor is not available except as part of the particulate enzyme preparation. Since GDP-D-[^{14}C]mannose is easily obtainable from commercial sources, the availability of an acceptor for the mannosyl transferase should facilitate the solubilization and purification of this enzyme.

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