METABOLIC RELATIONSHIPS BETWEEN PHOSPHO(GALACTO)LIPIDS IN *BIFIDOBACTERIUM BIFIDUM* VAR. PENNSYLVANICUS

F. W. van SCHAIK* and J. H. VEERKAMP
Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen, The Netherlands

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1. Introduction

Phosphoglycosyldiglycerides seem to be common membrane components of bacteria. Hitherto, about ten different phosphoglycolipids have been described (for review see [1]). *sn*-Glycero-1-phosphoryl galactofuranosyldiglyceride (fig.1) is the main phosphoglycolipid of *Bifidobacterium bifidum* var. pennsylvanicus. Its structure has been reported earlier [2,3]. The biosynthesis of the phosphogalactolipid may occur by transfer of the unusual *sn*-glycero-1-phosphate unit to galactofuranosyldiglyceride or its acyl-sugar derivative (fig.1). Both glycolipids are present in the bacterial membrane [4]. Phosphatidylglycerol, occurring abundantly in the membrane [5], may function as a donor of the *sn*-glycero-1-phosphate. A pathway in which CDP-diglyceride or CDP-glycerol is involved can be excluded, since these substrates would introduce a *sn*-glycero-3-phosphate moiety or its acylated analogue.

This report presents a study of pulse-chase experiments on intact cells, carried out to demonstrate a precursor-product relationship between phosphatidylglycerol (PG) and glycerophosphorylgalactosyldiglyceride (GPGalDG). ^{32}P-inorganic phosphate has been used as the labeling isotope.

The time-course of the specific activities of the phosphate-containing lipids agreed with the precursor-product relationship between PG and GPGalDG under conditions of rapid growth. When cell wall synthesis was inhibited with penicillin a preferential conversion of PG to diphosphatidylglycerol (DPG) occurred.

2. Materials and methods

B. bifidum var. pennsylvanicus was cultivated in Norris medium [6] containing 2% human milk as a growth factor essential for cell wall synthesis. The cultures were grown to the late log phase in 10.5 h. Then 1 mCi $^{32}P_i$ was added per culture. After a 60 min pulse the labeled cells were sedimented by centrifugation, subsequently resuspended in an equal

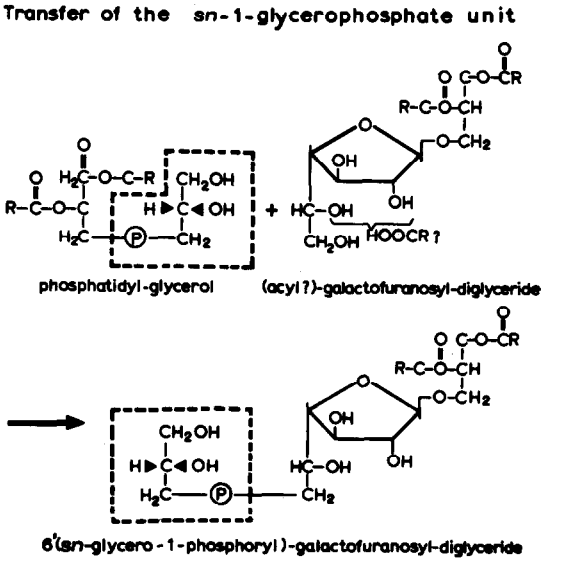

Fig.1. Transfer of the *sn*-glycero-1-phosphate moiety from PG to (acyl)-galactosyldiglyceride.

* Present address: Department of Physiology, Fac. Medicine, University of Utrecht, Vondellaan 24, Utrecht, The Netherlands.

volume of non-radioactive medium and cultivated again anaerobically at 37°C.

During the chase period samples were withdrawn from the culture at different times. Growth was measured as the increase of absorbance at 550 nm. The lipids were extracted from whole cells using a mixture of chloroform/methanol/buffer, as described previously [5]. Preparative one-dimensional chromatography was applied for the separation of the ^{32}P-containing lipids using silica gel HR and chloroform–methanol–acetic acid–water (125:37:9:9.5:1.5, by vol.) as developer. The lipids were located by scanning of the radioactivity with a Berthold thin-layer scanner. After elution from the silica gel the specific activity of the lipids was determined. Lipid-phosphorus was assayed according to Bartlett [7].

Inhibition of growth during chase was effected by a low pH (5.5) of the medium and by the presence of either penicillin (10 mg/l), chloramphenicol (60 mg/l) or bacitracin (400 mg/l culture). Omission of human milk was also investigated as a growth inhibitory condition.

The specific activity of $^{32}P_i$ in the cultures during standardized pulse-labeling amounted 1500 cpm/nmol. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter model 3380. ^{32}P-orthophosphate was purchased from Philips Duphar, Petten, The Netherlands.

The specific activity courses give information about the sequence of formation in a pathway as was first shown by Zilversmit et al. [8,9]. When the labeling isotope is withdrawn from the system prior to isotope equilibrium state, the specific activity of compound B equals that of its direct precursor A at the point where B has its maximal value. This precursor-product relationship will hold even when there is branching and reutilization as given in the next reaction sequences:

$$P_i \longrightarrow \longrightarrow \longrightarrow PG \longrightarrow GPGalDG$$
$$\uparrow\downarrow$$
$$DPG$$

3. Results

Cells grown to the late log phase were pulse-labeled during 60 min. The height of ^{32}P-incorporation into the phosphate-containing lipids appeared to be dependent upon the increase of cell mass (table 1). Overall-growth during the 60 min pulse varied from 0.10 to 0.55 duplication in different cultures. This range was due to variations of temperature when $^{32}P_i$ was added to the culture and the anaerobic condition was restored. In all cultures PG was much higher labeled than GPGalDG and DPG (table 1), which agrees with the idea that PG is formed earlier.

Cells grown for 0.10 duplication during labeling were resuspended in fresh, non-radioactive medium. The cell mass then increased twice in 3.5 h (fig.2a). The specific activity of PG and of GPGalDG are equal when the latter has its maximal value (fig.2b), suggesting that PG delivers the glycerophosphate moiety of the phosphogalactolipid under this condition. A precursor–product relationship between PG and DPG does not appear from the graph. Considerable ^{32}P-incorporation into DPG takes place during the first quarter of the chase period, but the specific activity curves of PG and DPG do not intersect when the latter has reached its maximum.

It seemed to us very possible that a conversion of PG to GPGalDG or to DPG is controlled by environmental factors. Therefore, we also followed the specific activity courses during chase under growth inhibitory conditions. Bacteria grown for 0.20 duplication during the 60 min pulse were resuspended in non-radioactive medium (pH 5.5) supplied with penicillin (fig.3). During the chase period overall-growth was fully inhibited. Initially, the specific activity of

Table 1
Dependence of ^{32}P-incorporation upon the viability of the cells

Growth (duplication)	Spec. activity (cpm/nmol P) PG	GPGalDG	DPG
0.10	275	95	35
0.20	530	140	140
0.25	490	170	180
0.55	810	460	330

The values were determined in different cultures after 60 min of pulse-labeling. Isotope equilibrium correspond to 1500 cpm/nmol P in all cultures. Abbreviations: PG, phosphatidylglycerol; GPGalDG, glycerophosphorylgalactosyldiglyceride and DPG, diphosphatidylglycerol (cardiolipin).

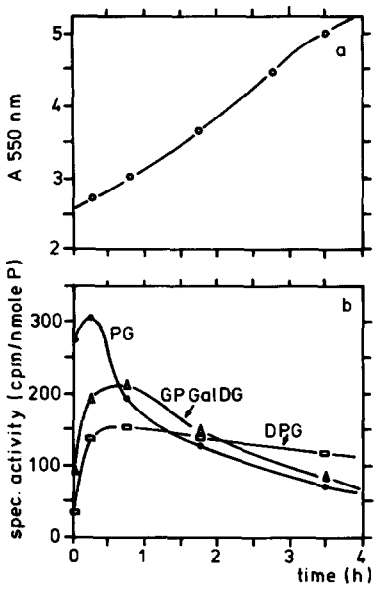

Fig.2. Chase of incorporated radioactivity during conditions of rapid growth. (a) absorbance of the culture; (b) specific activity courses of PG, GPGalDG and DPG. The abbreviations are given in table 1. The culture was grown for 0.10 duplication during the preceding 60 min pulse.

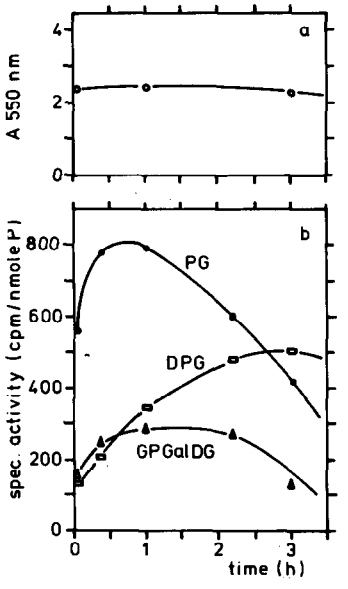

Fig.3. Chase of incorporated radioactivity during inhibitions of cell wall synthesis by penicillin. (a) absorbance of the culture; (b) specific activity courses. The culture was grown for 0.20 duplication during the preceding 60 min pulse.

PG increased considerably. This can be ascribed to the high specific activity of intracellular $^{32}P_i$ during the inhibition of growth. The curve of PG is decreasing thereafter, while the curve of DPG is still increasing. The curves intersect when DPG has its maximal value. The specific activity curves agree with a precursor-product relationship between PG and DPG, but not with one between PG and GPGalDG under this condition.

When growth was inhibited by the presence of bacitracin, or chloramphenicol or in the absence of human milk chase of incorporated radioactivity was evident only for PG, whereas the label in DPG and GPGalDG was quite stable. This implies, that PG is not or only to a small extent converted to DPG and GPGalDG under these conditions. The specific activity of PG probably decreased owing to deacylation, to conversion to other cellular components or to release into the medium.

4. Discussion

The synthesis of the phosphogalactolipid GPGalDG through a transfer of the *sn*-glycero-1-phosphate moiety of PG to galactosyldiglyceride is sustained by the chase under conditions of active growth. This transfer reaction may imply phospholipase C activity in the cells similar as the phosphatidylation reaction by phospholipase D [10].

The Zilversmit precursor–product relationship may be masked when the following conditions are not fulfilled. First, PG is the single compound that delivers its phosphate unit to the next product. Secondly, there must be an uniformity of distribution of the phospho(galacto)lipids in the membrane.

When DPG is formed through the CDP-diglyceride pathway, there are two direct precursors, CDP-diglyceride and PG, each yielding one phosphate unit. Although this pathway seems to be of minor importance in bacteria in contrast to that in eucaryotes [11,12], it can mask the precursor–product relationship in our experiment (fig.2b).

The reaction in which DPG is formed from two molecules of PG (2 PG→DPG + glycerol) seems to be common to bacteria [11–13]. In this reaction PG is the donor of both phosphate units in DPG. In *B. bifidum* var. pennsylvanicus DPG is probably also

formed by this non-energy requiring reaction, at least in the presence of penicillin, regarding the specific activity curves (fig.3b).

Cell wall inhibition brings about an increased net synthesis of DPG rather than of GPGalDG as was reported previously [5]. In the presence of penicillin, cycloserine, vancomycin or in the absence of human milk even a 5–8-fold increase was found for the cellular amount of DPG.

Inhomogeneous labeling of membrane phospholipids is now generally being recognized [14–16]. It is possible that separate topological entities of PG in the membrane are specialized either in the synthesis of DPG or GPGalDG. When both entities are labeled at different rates the precursor–product relationship would hold for this topological membrane areas but not for the combined pools. Furthermore, release of phospholipids into the medium can also disturb the precursor–product relationship when the excreted phospholipids originate preferentially from one pool. Release takes place indeed by inhibition of cell wall synthesis in *B. bifidum* var. pennsylvanicus [17].

Recently [18], more experimental evidence has been obtained for the synthesis of GPGalDG and DPG from PG by incubation of membrane preparations of our organism. Pieringer et al. [1,19] reported a similar reaction for the synthesis of a phosphoglycolipid using a membrane fraction of *Streptococcus faecalis*. In this reaction the phosphatidyl unit of PG or DPG, containing the *sn*-glycero-3-phosphate structure, was transferred forming phosphatidyldiglucosyldiglyceride.

Acknowledgements

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