BIOSYNTHESIS OF TEICHOIC ACID IN *MICROCOCCUS VARIANS* ATCC 29750

Characterization of a further lipid intermediate

H. A. I. McARTHUR, I. C. HANCOCK, F. M. ROBERTS* and J. BADDILEY
Microbiological Chemistry Research Laboratory, The University, Newcastle upon Tyne NE1 7RU, England

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1. Introduction

Attachment of the poly(ribitol phosphate) teichoic acids of *Staphylococcus aureus* and *Bacillus subtilis* and the poly(*N*-acetylglucosamine 1-phosphate) of *Micrococcus varians* ATCC 29750 to peptidoglycan is achieved through a linkage unit of *N*-acetylglucosamine 1-phosphate-tri(glycerol phosphate) [1–9]. The biosynthesis of linkage unit, from its precursors UDP-*N*-acetylglucosamine (UDP-GlcNAc) and CDP-glycerol, involves the participation of a series of lipid intermediates as follows:

The intermediates are believed to be polyprenyl pyrophosphate derivatives. Evidence for this comes from earlier studies on the chemistry of lipids labelled during teichoic acid biosynthesis [10,11], the interdependence of peptidoglycan and teichoic acid synthesis and their inhibition by bacitracin [12,13], and more recently the sensitivity of linkage unit synthesis to the antibiotic tunicamycin [4–7]. Tunicamycin inhibits specifically the transfer of *N*-acetylglucosamine 1-phosphate from its uridine nucleotide to polyprenyl phosphate [14], the initial reaction (a) in the scheme.

(a) Prenyl-P + UDP-GlcNAc →
UMP + Prenyl-PP-GlcNAc (lipid I)

(b) Prenyl-PP-GlcNAc + CDP-Glycerol →
CMP + Prenyl-PP-GlcNAc-P-glycerol (lipid II)

(c) Prenyl-PP-GlcNAc-P-glycerol + CDP-Glycerol →
CMP + Prenyl-PP-GlcNAc-$(P\text{-glycerol})_2$ (lipid III)

(d) Prenyl-PP-GlcNAc-$(P\text{-glycerol})_2$ + CDP-Glycerol →
CMP + Prenyl-PP-GlcNAc-$(P\text{-glycerol})_3$ (lipid IV)

(e) Prenyl-PP-GlcNAc-$(P\text{-glycerol})_3$ + 30 CDP-Ribitol →
or
+ 20 UDP-GlcNAc
30 CMP + Prenyl-PP-GlcNAc-$(P\text{-glycerol})_3$-$(P\text{-ribitol})_{30}$
or or
20 UMP -$(P\text{-GlcNAc})_{20}$

(f) Prenyl-PP-GlcNAc-$(P\text{-glycerol})_3$-$(P\text{-ribitol})_{30}$ + Peptidoglycan →
Prenyl-P + Peptidoglycan-P-GlcNAc-$(P\text{-glycerol})_3$-$(P\text{-ribitol})_{30}$

* Present address: Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

Assembly of the main teichoic acid chain occurs from its appropriate precursor and it is attached through a phosphodiester bond to the terminal glycerol phosphate of the linkage unit. It is unclear whether this reaction proceeds in vivo via lipoteichoic acid carrier (LTC) [15] or by direct transfer from nucleotides on to polyprenyl-PP-GlcNAc-$(P\text{-glycerol})_3$ (lipid IV); both reactions have been demonstrated in vitro [16,17]. The completed teichoic acid-linkage unit is then transferred to nascent peptidoglycan by the formation of a phosphodiester bond between the 1-position of *N*-acetylglucosamine and the 6-hydroxyl group of a muramic acid residue of peptidoglycan [18,19]. We have described the characterization of lipids I, II and III, and have shown them to be sequential intermediates on the pathway of linkage unit biosynthesis [7,8]. Additionally, it has been shown that membrane preparations from *Micrococcus varians* catalyse the de novo synthesis and transfer of poly(*N*-acetylglucosamine 1-phosphate) attached to linkage unit, to concomitantly synthesized non-crosslinked peptidoglycan [19]. However, we were unable to identify a polyprenyl pyrophosphate intermediate containing 3 glycerol phosphate residues, although it was demonstrated that all three glycerol phosphate residues in the linkage unit are derived from CDP-glycerol [8].

Here we report the isolation and identification of the putative lipid IV, polyprenyl–PP–GlcNAc–$(P\text{–glycerol})_3$.

2. Materials and methods

CDP-[U-^{14}C]Glycerol (174 mCi/mmol) prepared from D-[U-^{14}C]glycerol 1-phosphate, CDP-[2-^{3}H]-glycerol (580 mCi/mmol) prepared from [2-^{3}H]-glycerol and CDP-glycerol were made as in [13]. UDP-*N*-acetylglucosamine (UDP-GlcNAc), glucosamine hydrochloride and glucosamine 6-phosphate were purchased from the Sigma Chemical Co. (Poole, Dorset). Glucosaminitol and its 6-phosphate were synthesized by the method in [20].

Small-scale reaction mixtures contained 50 μl membrane suspension, from *S. aureus* H or *M. varians* ATCC 29750 prepared as in [7], 20 mM magnesium acetate, and in the case of *S. aureus* H 1.7 mM ATP. Additions were made where required to the following concentrations in 100 μl final vol: CDP-[^{14}C]-glycerol, 0.042 M (1.6×10^5 dpm); CDP-[^{3}H]glycerol, 0.025 mM (10^6 dpm); UDP-GlcNAc, 1 mM; tunicamycin (a gencrous gift from Professor G. Tamura), 10 μg/ml. Incubation was at 30°C for the appropriate period; the reaction was terminated by immersion in boiling water for 2 min.

Paper chromatography was carried out on Whatman 3MM paper in solvents A, isobutyric acid–0.5 M aq. ammonia (5:3, v/v); B, propan-1-ol-aq. ammonia (0.88 sp.gr.)–water (6:3:1, by vol.); C. butan-1-ol–pyridine–water (6:4:3, by vol.). Electrophoresis was performed on Whatman no. 1 paper in 0.06 M $Na_2B_4O_7$–NaOH (pH 9.5) at 40 V/cm for 2.5 h [21].

Radioactivity on paper was determined using toluene scintillation fluid [13]. Radioactivity in aqueous solution was measured (100 μl samples) in 3 ml scintillant containing diphenyloxazole (0.4%, w/v) in toluene–Triton X-100 (1:1, v/v). Dual-label counting efficiency was 8.5% for ^{3}H and 31.2% for ^{14}C on paper and 28.8% for ^{3}H and 44.6% for ^{14}C in solution. The overlap of ^{14}C into ^{3}H was 43.5% on paper and 65.0% in solution.

Sodium boro[^{3}H]hydride (6.24 Ci/mmol, 100 mCi/ml) was dissolved in freshly-distilled anhydrous dimethylformamide [21]. Samples were reduced with 0.5 ml alkaline sodium boro[^{3}H]hydride solution. This was prepared by mixing 200 μl sodium boro[^{3}H]hydride in dimethylformamide with 2.8 ml 25 mM NaOH. The reaction was incubated at 30°C for 16 h and terminated by the addition of 100 μl acetic acid. The mixtures were evaporated to dryness, the residues dissolved in 0.5 ml 1 M acetic acid, and again evaporated to dryness. The evaporation with 1 M acetic acid was repeated 5 times to ensure the removal of exchangeable ^{3}H.

Acid and alkaline hydrolyses and treatment with alkaline phosphatase were as in [7]. D-[U-^{14}C]glycerol 1-phosphate, [2-^{3}H]glycerol, D-[U-^{14}C]glucose (316 mCi/mmol) and sodium boro[^{3}H]hydride were purchased from The Radiochemical Centre, Amersham.

3. Results

In [7,8] we had been unable to detect a lipid intermediate containing 3 glycerol phosphate residues in either *S. aureus* H or *M. varians*. However, when UDP-GlcNAc and CDP-[^{3}H]glycerol were incubated with an excess (200 μl) of a concentrated membrane suspension (40–45 mg protein/ml) from *S. aureus* H, an additional radioactive product (R_F

0.15) was detected. In conjunction with the radioactive material identified in [7] as lipids II and III (R_F 0.77 and 0.66, respectively), formation of the new product required UDP-GlcNAc and its synthesis was inhibited by tunicamycin (fig.1).

Even with the large amounts of membrane used in the incubation mixture, little of the novel product was formed. However, much larger quantities of lipid intermediates are obtained, for an equivalent weight of membranes, from *M. varians* than from *S. aureus*

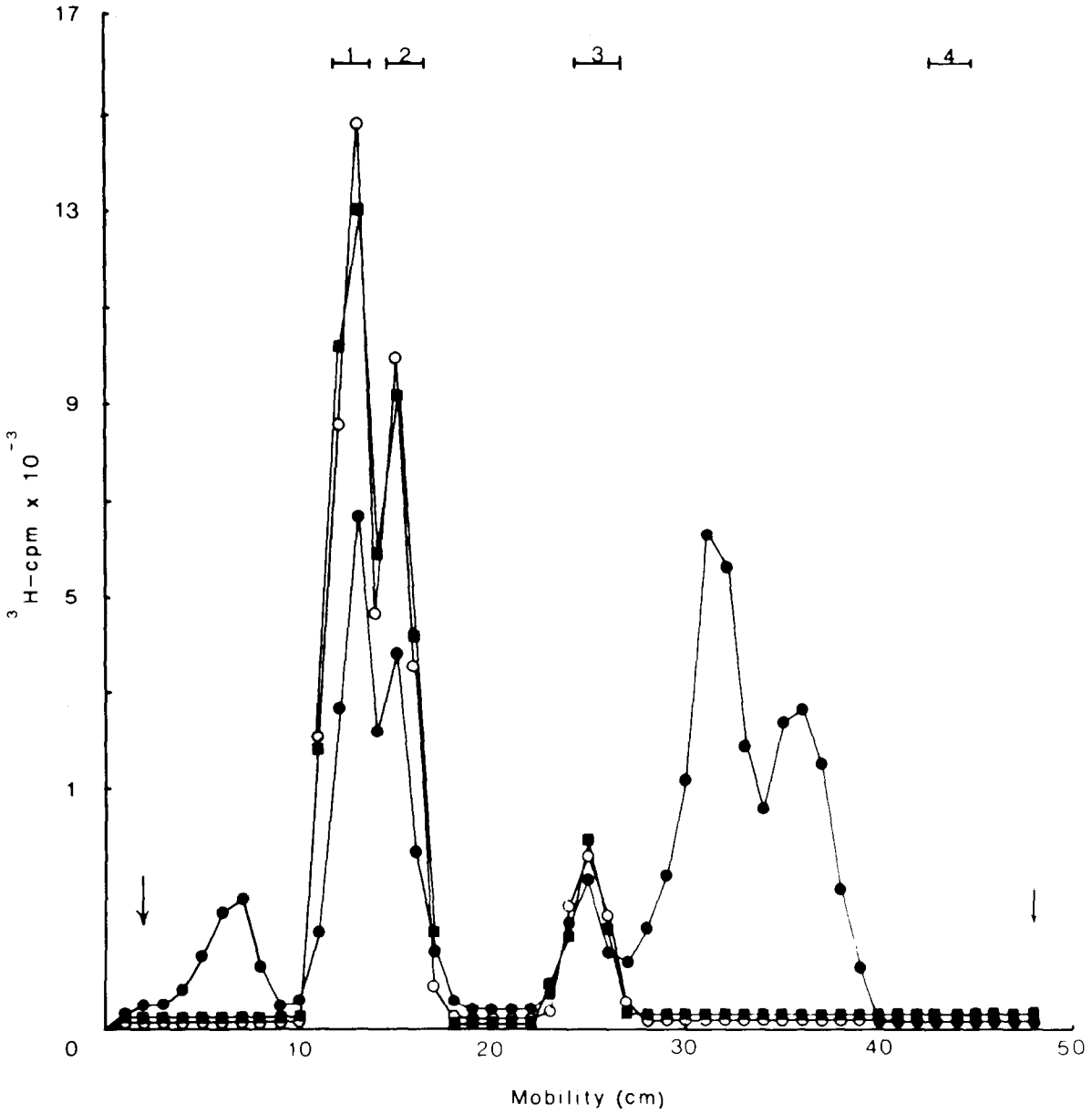

Fig.1. Synthesis of linkage unit intermediates in *S. aureus* H. Membranes (200 μl, 45 mg protein/ml) were incubated with CDP-[^{3}H]glycerol in the presence (•) or absence (○) of UDP-GlcNAc at 30°C for 30 min. Tunicamycin (10 μg/ml) was included in the former incubation (▪). Reaction products were separated by chromatography in solvent A. Markers indicate the location of: (1) CDP-glycerol; (2) glycerol phosphate; (3) glycerol; (4) phosphatidylglycerol. Arrows show the origin and solvent front, respectively.

[8], but the requirement for UDP-GlcNAc as the precursor of poly(*N*-acetylglucosamine 1-phosphate) in the former organism precludes the synthesis of linkage unit intermediates without concomitant synthesis of main chain. As it seemed likely that the isolation of the new lipid would be enhanced if simultaneous synthesis of the main chain could be avoided *M. varians* membranes were preincubated with UDP-GlcNAc, the residual substrate removed by centrifugation, and the membranes reincubated with CDP-[^{14}C]glycerol (table 1). If UDP-GlcNAc was omitted from the preincubation, a small amount of radioactivity from CDP-[^{14}C]glycerol was incorporated into lipids II and III and into polymeric material at the origin of the chromatogram. This represents the formation of the glycerol-containing linkage unit intermediates from endogenous lipid I, and its subsequent transfer to pre-existing poly(*N*-acetylglucosamine 1-phosphate) synthesized on an acceptor similar to LTC [8,17]. In contrast, preincubation with UDP-GlcNAc resulted in the formation of large amounts of lipids II and III, and a considerable accumulation of the product with R_F 0.15, on subsequent incubation with CDP-[^{14}C]-glycerol; there was some increase in the amount of linkage unit attached to poly(*N*-acetylglucosamine 1-phosphate). If UDP-GlcNAc and CDP-[^{14}C]glycerol were both included in the second incubation most of the radioactivity derived from the substrate was incorporated into polymer-associated linkage unit; the amounts of lipids II and III were decreased, while little or none of the new lipid was formed.

When tunicamycin was present during the initial incubation, the transfer of radioactivity was observed solely to endogenous linkage unit intermediates and poly(*N*-acetylglucosamine 1-phosphate). However, tunicamycin had no effect when included only in the second incubation; thus the antibiotic does not prevent the transfer of glycerol phosphate residues to lipid I accumulated during the initial incubation.

Hence, it seemed likely that the product with R_F 0.15 was a new linkage unit intermediate. Maximal accumulation of the product occurred after 30 min of the second incubation and attempts were made to isolate and characterize it. Large scale incubation mixtures (1.5 ml membranes) were prepared as described in table 1, and the components separated by chromatography in solvent A. Like other linkage unit intermediates, the new product could be extracted from the paper with 70% (v/v) ethanol [6,8,16]. It was hydrolysed with NaOH (0.5 M, 20°C, 2 h) and subjected to chromatography on Sephadex G-50 (1.6 × 50 cm). The radioactive material emerged as a single peak at 65–75 ml. After concentration the ^{14}C-labelled product chromatographed as a single compound in solvent B with $R_{glycerolphosphate}$ (R_{GP}) 0.9. Treatment with alkaline phosphatase yielded a

Table 1
Synthesis of lipid intermediates and the effect of tunicamycin

Incubation 1	Tunicamycin (10 μg/ml)	Incubation 2	Tunicamycin (10 μg/ml)	^{14}C (cpm)			
				Linkage unit attached to polymer	Lipid IV (R_F 0.15)	Lipid III	Lipid II
None	–	CDP-[^{14}C]glycerol	–	1270	0	425	370
			+	1430	0	380	440
UDP-GlcNAc	–	CDP-[^{14}C]glycerol	–	2860	1835	10 850	6725
	+		–	1145	0	320	295
	–		+	3250	1740	11 485	6480
None	–	UDP-GlcNAc CDP-[^{14}C]glycerol	–	15 320	465	4810	2650
[a]	–		–	70	0	0	0
	–		+	1340	0	280	300

[a] Membranes were boiled for 2 min prior to the first incubation

The initial incubation mixture contained 200 μl membranes, and the additions indicated in 250 μl total vol. After incubation at 30°C for 20 min the membranes were washed by centrifugation with 22 ml 0.05 M Tris–HCl, 10 mM Mg^{2+} (pH 8.0) at 125 000 × *g* for 45 min. The washed membranes were resuspended to 200 μl in 0.05 M Tris–HCl (pH 8.0); duplicate 100 μl samples were then reincubated as shown for a further 30 min

Table 2
Sodium boro[^{3}H]hydride reduction

Lipid IV	^{14}C (dpm)	^{3}H	^{14}C (pmol)	^{3}H	Ratio (^{14}C/^{3}H)	
a	20 363	58 641	53.20	19.00	2.80	
b	33 170	91 273	86.65	29.57	2.93	
Polymer fragment						
a	42 181	137 217	110.19	44.46	2.48	
b	52 056	165 982	135.99	53.78	2.53	
Glucose	^{14}C (dpm)	^{3}H	Ratio (^{3}H/^{14}C)		Spec. act. glucose	Spec. act. ^{3}H (Ci/mmol^{-1})
a	56 661	252 823	4.462		0.316	1.410
b	41 168	181 868	4.418		0.316	1.396

Products obtained after controlled acid hydrolysis (0.1 M HCl, 100°C for 10 or 15 min, respectively) of lipid IV and linkage unit attached to polymer (see table 1) were reduced with sodium boro[^{3}H]hydride; the extent of reaction (1.403 Ci/mmol) was determined by the concomitant reduction of D-[U-^{14}C]glucose (316 mCi/mmol); products were separated by chromatography in solvent C

product with identical mobilities to authentic bis(glycerophosphoryl)glycerol (prepared by deacylation of cardiolipin [22]), in solvent B (R_{GP} 1.6) and in Sephadex G-50 (75–95 ml).

Hydrolysis of the unknown product (0.1 M HCl, 100°C, 10 min) followed by chromatography on Sephadex G-50, gave a single peak at 50–70 ml, with R_{GP} 1.0–1.1 in solvent B. An equivalent fragment was isolated from [^{14}C]glycerol-labelled linkage unit attached to poly(*N*-acetylglucosamine 1-phosphate), by hydrolysis of material at the origin of the chromatogram (0.1 M HCl, 100°C, 15 min); it was eluted at the same position on Sephadex G-50 and had a similar mobility in solvent B. These samples were reduced with sodium boro[^{3}H]hydride and purified by chromatography on Sephadex G-50 and in solvent B. The results are shown in table 2. The reduced fragments were hydrolysed (2 M HCl, 100°C, 3 h) and the acid was removed in vacuo over KOH pellets. In each instance 70–80% of the ^{3}H was associated with a compound with the same mobility as glucosaminitol phosphate (R_{GP} 0.78) in solvent B, and electrophoresis at pH 9.5 ($m_{D\text{-glucitol}}$ 1.35). Treatment with alkaline phosphatase gave a compound that co-chromatographed with glucosaminitol (R_{GP} 2.10; $m_{Glucitol}$ 0.45). The remainder of the ^{3}H had the same mobility as glucosaminitol; it was unaffected by alkaline phosphatase. ^{14}C was associated with glycerol monophosphate(s), glycerol diphosphate(s) and glycerol.

4. Discussion

The synthesis of the new product (R_F 0.15) was dependent upon UDP-GlcNAc and was inhibited by tunicamycin. Alkaline hydrolysis (fig.2) of the product yielded the tri(glycerol phosphate) moiety in an analogous manner to that obtained in [8]. After controlled acid hydrolysis and reduction with sodium boro[^{3}H]hydride, the ^{14}C to ^{3}H ratio in the product was almost 3:1. All recovered ^{3}H was present in glucosaminitol phosphate or glucosaminitol, whereas the ^{14}C was in glycerol and its mono- and diphosphate(s). These results are identical with those obtained by degradation of the linkage unit in cell walls [23]. Thus the product had the properties expected of the putative lipid IV and must have the structure polyprenyl PP-GlcNAc-(P-glycerol)$_3$. Although fully characterized only in *M. varians*, it is concluded that lipid IV with the same structure is present in *S. aureus* H.

The transfer of a third glycerol phosphate residue to lipid III resulted in a considerable decrease in mobility in solvent A (0.66 to 0.15). This indicates a substantial increase in hydrophilicity on the attachment of the additional residue; this effect is greater than that shown by hexosyl residues in the linkage unit of the teichuronic acid of *Micrococcus lysodeikticus* [24].

Control of teichoic acid synthesis has been

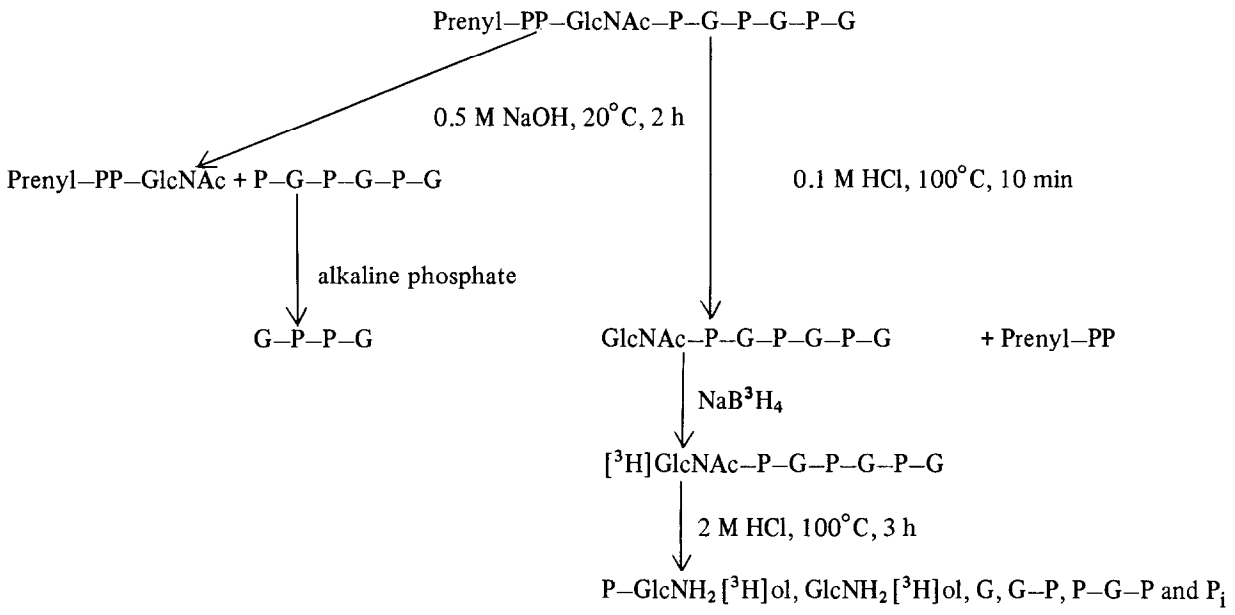

Fig.2. Scheme for the degradation of polyprenyl-PP-GlcNAc(glycerol phosphate)$_3$. GlcNH$_2$-ol and P-GlcNH$_2$ol represent glucosaminitol and glucosaminitol phosphate, respectively; G, glycerol.

observed on CDP-glycerol pyrophosphorylase [25], and on the formation of lipid I [26,27]. However, as lipid IV represents the intermediate to which the main chain is attached, it is reasonable to postulate some control of its synthesis or utilization. Accumulation of lipid IV was greatest when synthesis of linkage unit could occur in the presence of pre-existing main chain attached to LTC. Transfer of linkage unit to main chain is observed under these conditions [17].

Our investigations have also shown that main chain can be attached to incomplete linkage unit intermediates in vitro [8,17]. This is reflected in the ratio of ^{14}C to ^{3}H in the fragment isolated by controlled acid hydrolysis of linkage unit attached to main chain (table 2). Although some decrease in the 3:1 ratio could be expected from the transfer of [^{14}C]glycerol phosphate to endogenous lipids II and III, the amount of polymer-attached linkage unit formed under such conditions is <10% of that obtained when both main chain and linkage unit synthesis occur concomitantly (table 1). Thus the decrease of the ^{14}C to ^{3}H ratio in fragments to ~2.5 represents the transfer of main chain to incomplete linkage unit.

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