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INCORPORATION OF LABELLED GLYCEROLS INTO ETHER LIPIDS IN *CALDARIELLA ACIDOPHILA*

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Key Word Index—*Caldariella acidophila*; archaebacteria; thermophilic bacteria; isoprenoid biosynthesis; ether lipids.

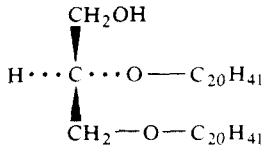
Abstract—The lipids of *Caldariella acidophila*, an extreme thermophile member of the new archaebacteria group, are macrocyclic tetraethers. They are made up of two glycerol molecules (or one glycerol and one nonitol) bridged through ether linkages by two C_{40}16,16′-biphytanyl chains. To elucidate the biosynthesis of the glycerol moiety of these tetraethers and the mechanism of glycerol ether assembly, labelled [U-^{14}C, 1(3)-^{3}H]glycerol and [U-^{14}C, 2-^{3}H]glycerol, were fed to *C. acidophila*. Both precursors were selectively incorporated with high efficiency, and without any change in the ^{3}H/^{14}C ratio, in the glycerol moiety of tetraethers. These results suggest that the ether forming step in the biosynthesis of tetraether lipids of *C. acidophila*, occurs without any loss of hydrogen from any of the glycerol carbons which in turn could be directly alkylated by geranylgeranyl pyrophosphate. The incorporation of radioactivity in the isoprenoid chains and into nonitol is also analysed.

INTRODUCTION

Archaebacteria, which at present comprise a considerable variety of methanogenic, halophilic and thermoacidophilic species has been separated from the classically recognized bacteria on grounds which imply very prolonged evolutionary separation [1]. One characteristic common to all is that their cell membrane is based upon ether lipids which, irrespective of a complex lipids structure, are derived from the three structural types **1–3**. The membrane lipids of the extreme halophiles are based on glycerol diether derivatives (see **1**) that have two aliphatic C_{20} chains with a phytanyl structure [2, 3]. The tetraethers **2** were first found in some thermoacidophilic archaebacteria, such as *Caldariella acidophila*, *Sulfolobus acidocaldarius* and *Thermoplasma acidophila* [4–6]. These tetraethers are based on five types of C_{40} isoprenoids (–$C_{40}H_{80}$–, –$C_{40}H_{78}$–, –$C_{40}H_{76}$–, –$C_{40}H_{74}$–, –$C_{40}H_{72}$–), which are made up to two phytanyl units joined head-to-head; they differ in as much as four cyclopentane rings. These structures have already been completely characterized by De Rosa *et al.*[7].

It has recently been reported that the archaebacterial methanogens are also characterized by the occurrence of ether lipids, and while some contain only diether lipids based on the ether **1** others contain the two ethers **1** and **2** [9–11]. The tetraethers **3**, which are found only in the extreme thermoacidophiles *C. acidophilia* and *S. acidocadarius*, are similar to the tetraether structures **2**, while one glycerol is substituted by a unique branched chain nonitol, named calditol [8]. All these glycerol ethers contain a 2,3-di-*O*-*sn*-glycerol [2, 4, 8] which is unusual since the glycerol in the naturally occurring glycerophosphatides or diacylglycerols is known to have a *sn*-1,2 stereochemistry.

Given the unusual nature of these archaebacterial lipids which have many structural analogies (**1–3**) it appeared of interest to study the mechanism of glycerol ether formation, which is one of the most important biochemical steps common to the biosynthesis of all archaebacterial lipids. The formation of the glycerol diether was first investigated by Kates *et al.* [12] in *Halobacterium cutirubrum*, an obligate halophilic bacterium. In incorporation experiments using differently labelled glycerols as precursors, they found that in the glycerol moiety of the diether lipids of *H. cutirubrum* (**1**), the label from [U-^{14}C, 1(3)-^{3}H]glycerol was incorporated intact, with 100% retention of the initial ^{3}H/^{14}C ratio. In contrast the label from [U-^{14}C, 2-^{3}H]glycerol was incorporated with an almost complete loss of ^{3}H. These authors regarded the glycerol β-carbon loss of tritium, during glycerol diether formation, as a significant feature of the etherification step. They suggested a more likely precursor would be dihydroxyacetone which could be formed by the action of glycerol dehydrogenase, an enzyme known to be active in *H. curitubrum* [2, 3]. Moreover they excluded the participation of aldo–keto isomerization, such as that between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, in the glycerol utilization for the glycerol moiety of diether lipids. To establish if Kates' conclusions on glycerol ether assembly are applicable to archaebacteria we have studied this biogenetic step in *C. acidophila*, an extreme acidothermophilic archaebacterium.



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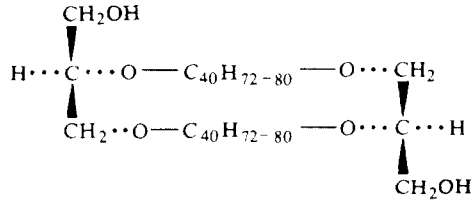

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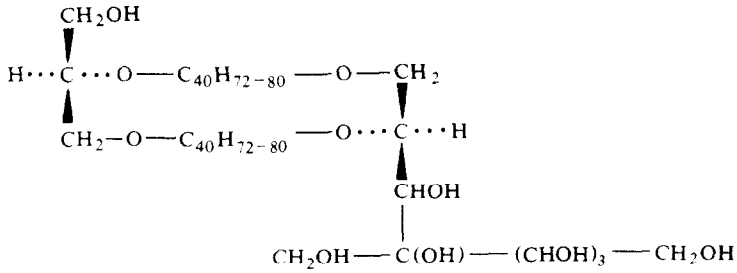

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RESULTS

In two separate experiments, growing cultures of *C. acidophila* were incubated with [U-^{14}C, 1(3)-^{3}H]glycerol and with [U-^{14}C, 2-^{3}H]glycerol. The lipids were extracted from the harvested cells and hydrolysed to obtain the two classes of macrocyclic tetraethers **2** and **3**, which are the basic components of all the complex lipids of the micro-organism.

Table 1 indicates the amount of radioactivity incorporated in the whole cells and in the lipids. The similarity of cell yields in the two experiments ensures the comparability of incorporations of the two differently labelled precursors.

While the total cellular ^{14}C radioactivity recovered from the cells and from the crude lipid fraction was practically the same in the two experiments, the retention of ^{3}H depended on the position of ^{3}H label in the glycerol precursor. The lipids of the cells grown in the presence of [U-^{14}C, 1(3)-^{3}H]glycerol had a higher retention of ^{3}H (63%) than the lipids from the cells of the experiment with [U-^{14}C, 2-^{3}H]glycerol (22%). To investigate in greater detail the role of

Table 1. Incorporation of labelled precursors into whole cells and into a crude lipid fraction

		Precursors	
	[U-^{14}C, 1(3)-^{3}H]glycerol	^{14}C 69 mCi/mmole ^{3}H 207 mCi/mmole	[U-^{14}C, 2-^{3}H]glycerol
Cells			
Yields (mg)*	84		80
10^{-6} × total radioactivity	^{14}C 23.2		25.4
(dpm)	^{3}H 51.1		21.0
Lipids			
Yields (g)†	3.0		3.1
10^{-6} × total radioactivity	^{14}C 12.4		12.7
(dpm)	^{3}H 23.5		8.5

*The yields refer to the amount of freeze-dried cells recovered from 340 ml of incubation expts with labelled precursors, see the Experimental.

†The yields refer to the amount of lipids extracted from labelled lyophilized cells, diluted 364 fold with unlabelled lyophilized cells.

The labelled precursors [U-^{14}C, 2-^{3}H]glycerol and [U-^{14}C, 1(3)-^{3}H]glycerol were added to the culture (340 ml) of *C. acidophila* at the beginning of the exponential phase. The cells were then harvested in the late-exponential phase and after a 364-fold dilution with unlabelled cells the lyophilized material was extracted with chloroform–methanol (1 : 1) for lipid recovery.

Table 2. Distribution of radioactivity in glycerol-dialkyl-glycerol and glycerol-dialkyl-calditol tetraethers

Compounds	Precursors $^3H/^{14}C = 3$ [U-^{14}C, 1(3)-^{3}H]glycerol Radioactivity (10^{-3} × dpm/mmol)			Precursors $^3H/^{14}C = 3$ [U-^{14}C, 2-^{3}H]glycerol Radioactivity (10^{-3} × dpm/mmol)		
	^{14}C	^3H	$^3H/^{14}C$	^{14}C	^3H	$^3H/^{14}C$
Glycerol-dialkyl-glycerol tetraethers	5880	15400	2.62	6000	14600	2.43
C_{40} Dichlorides from glycerol-dialkyl-glycerol tetraethers	590	830	1.41	631	90	0.14
Glycerol from glycerol-dialkyl-glycerol tetraethers	2310	6930	3.00	2348	7000	2.98
Glycerol-dialkyl-calditol tetraethers	10600	25000	2.36	11200	7440	0.66
C_{40} Dichlorides from glycerol-dialkyl-calditol tetraethers	630	870	1.38	650	95	0.15
Glycerol from glycerol-dialkyl-calditol tetraethers	2100	6200	2.95	2300	6900	3.00
Calditol from glycerol-dialkyl-calditol tetraethers	7340	15488	2.11	7438	240	0.03

For the experimental conditions see Table 1. Glycerol-dialkyl-glycerol tetraethers (**2**) and glycerol-dialkyl-calditol tetraethers (**3**) were obtained from lipids of *C. acidophila* by methanolic HCl hydrolysis. Degradation products of both tetraethers: C_{40} dichlorides, glycerol and calditol, were obtained by BCl_3 cleavage.

glycerol in the various metabolic events of lipid biosynthesis in *C. acidophila*, the labelled lipids were degraded as reported in the Experimental. Table 2 summarizes, for the two experiments, the distribution of radioactivity into the macrocyclic tetraethers (**2**, **3**). The $^3H/^{14}C$ ratio of the glycerol-dialkyl-glycerol tetraethers (**2**), obtained from the lipids of cells grown with the two differently labelled glycerols, decreases only slightly; this decrease was more evident with [U-^{14}C, 2-^{3}H]glycerol. The analysis of radioactivity distribution in these tetraethers, based on BCl_3 cleavage showed that the glycerol moieties of the glycerol-diaklyl-glycerol tetraethers (**2**), with both double-labelled precursors, incorporated the radioactivity without any change in the $^3H/^{14}C$ ratio and with a high efficiency. In fact, considering the 364-fold dilution of the labelled cells, the dilution of both glycerol precursors in the glycerol moiety of glycerol-dialkyl-glycerol tetraethers was only 181-fold.

Conversely the C_{40} isoprenoids $^3H/^{14}C$ ratio depended strictly on the ^3H position in the precursor; while [U-^{14}C, 1(3)-^{3}H]glycerol labelled the isoprenoids with 50% of ^3H recovery, ^3H was absent from the [U-^{14}C, 2-^{3}H]glycerol incorporation experiment.

The $^3H/^{14}C$ ratio in the glycerol-dialkyl-calditol tetraethers (**3**) also depended on the position of the ^3H label in the precursor, with [U-^{14}C, 2-^{3}H]glycerol, the ^3H retention was *ca* 22%, while with [U-^{14}C, 1(3)-^{3}H]glycerol, it was 79%.

The analysis of the radioactivity distribution in this case showed that the molar radioactivity and $^3H/^{14}C$ ratio of the glycerol and the C_{40} isoprenoids, with both glycerol precursors were, as expected, similar to that observed in the same part structures of the glycerol-dialkyl-glycerol tetraethers (**2**). Furthermore with [U-^{14}C, 1(3)-^{3}H]glycerol, *ca* 70% of ^3H (Table 2) was recovered in the calditol moiety while with [U-^{14}C, 2-^{3}H]gylcerol, ^3H labelling was absent.

Finally it is interesting to note that the $^3H/^{14}C$ ratio of the glycerol–glycerol phosphate pool of cells grown in the presence of [U-^{14}C, 2-^{3}H]glycerol and [U-^{14}C, 1(3)-^{3}H]glycerol were respectively 2.97 and 3.02; these values were about the same as the supplied precursors.

DISCUSSION

Our results go towards clarifying the mechanism underlying glycerol ether assembly in *C. acidophila*. The unequivocal rationalization of incorporation data on double labelled glycerols requires the evaluation of the eventual role of isotopic effects due to the discriminatory capability against ^3H by enzymes involved in glycerol utilization.

In previous papers [19, 20] on glycerol lipid biosynthesis, performed by incorporation of double labelled glycerols, the authors reported a significant isotopic effect of *sn*-glycero-3-phosphate dehydrogenase (EC 1.1.99.5) which discriminates against *sn*-2-^{3}H glycerol-3-phosphate during the oxidation. This effect, causing an increase of the $^3H/^{14}C$ ratio in the *sn*-glycerol-3-phosphate pool, represents a limit in the correct evaluation of incorporation data.

Our results on the $^3H/^{14}C$ ratio of the glycerol–glycerophosphate pool in *C. acidophila* cells, at the end of incubation experiments with double labelled glycerols, show that the enzymes directly involved in the glycerol utilization are not able to discriminate

against ^3H. In fact in both cases the isotope ratio of the glycerol–glycerol phosphate pool does not change, with respect to the original values of the fed precursors.

As expected from the normal glycolytic pathway, while [U-^{14}C, 1(3)-^3H]glycerol gave substantial ^3H retention in the phytanyl chains via acetyl-CoA, and also in the sugar of complex lipids via triose, [U-^{14}C, 2-^3H]glycerol gave rise to a loss of ^3H label in the bisphytanyl C_{40} chains and in the lipid sugars. These results are further evidence of a classical mevalonate pathway in archaebacteria, as previously demonstrated by incorporation studies of more direct precursors such as acetate and mevalonate [2, 4, 21].

In relation to the mechanism of the glycerol ether assembly in *C. acidophila*, the results on labelling of glycerol moieties into tetraethers **2**, and **3** show that labelled glycerol precursors were in both cases incorporated intact, with a 100% conservation of the initial $^3H/^{14}C$ ratio. Thus the ether-forming step occurs without any loss of hydrogen from any of the glycerol carbons and therefore without the intervening formation of any oxidized intermediate derivative of the glycerol. Given the well demonstrated ability of geranylgeranyl and similar allylic pyrophosphates to act as alkylating agents in other biosynthetic mechanisms, direct ether formation from glycerol (or, facilitated by neighbouring group deprotonation, from glycerol phosphate) presents no conceptual difficulties.

On the other hand such alkylating reactivity would be lessened in a non-allylic (phytanyl) pyrophosphate, and we regard this as evidence that ether formation could precede reduction in the isoprenoid part of ether lipids in *C. acidophila*. According to this hypothesis the unusual configuration of the chiral centre in the glycerol moiety would depend, in this microorganism, on the stereospecific nature of the alkylation step.

The proposed mechanism of glycerol ether formation in *C. acidophila* differs from that suggested by Kates *et al.* [12], who obtained both direct and presumptive evidence for a general pathway of glycerol metabolism in halobacteria. Although their conclusions are fully consistent with their observations, they throw no light on the mechanisms of ether lipid formation. In fact these authors regarded the loss of ^3H from the β-carbon of glycerol, in glycerol diether formation, as a significant feature of the etherification step, while our results with *C. acidophila* favour the alternative explanation that in halobacteria the β-carbon ^3H loss is probably due to an efficient interconversion of glycerol (and/or glycerophosphate and dihydroxyacetone and/or phosphate).

The observation that [U-^{14}C, 1(3)-^3H]glycerol labels the calditol moiety, with 70% ^3H retention, while [U-^{14}C, 2-^3H]glycerol was incorporated with a complete loss of ^3H, and preliminary evidence of a selective localization of the radioactivity, with both precursors, at the level of carbons 1–3 of calditol (**3**) (experimental detail not given), favours a biosynthetic route that implies first the oxidation of the secondary carbon of glycerol and then the removal of one hydrogen from this oxidized intermediate in the assembly of the C_9 calditol skeleton.

EXPERIMENTAL

Radiochemicals. [U-^{14}C]Glycerol (119 mCi/mmole), [1(3)-^3H]glycerol (0.5 Ci/mmole) and [2-^3H]glycerol (0.5 Ci/mmole) were purchased from The Radiochemical Centre, Amersham. The double labelled precursors [U-^{14}C, 1(3)-^3H]glycerol and [U-^{14}C, 2-^3H]glycerol were prepared by mixing the corresponding single labelled glycerols, to obtain a $^3H/^{14}C$ ratio of 3.

Micro-organism and culture conditions. C. acidophilia, strain MT-4, isolated from an acidic hot spring in Agnano, Naples [13], was grown at 87° without shaking. The culture medium contained (per l.) 1 g yeast extract, 1 g casamino acids, 3.1 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.25 g $CaCl_2 \cdot H_2O$, the pH was adjusted to 3 with 0.1 M H_2SO_4 and the culture vessel was inoculated by adding a 12 hr broth culture (0.1 l. for 1 l. of medium). The double labelled precursor was added to the culture (300 ml) at the beginning of the exponential phase. Cells were harvested in the late stationary growth phase of incubation (48 hr) by centrifugation at 33000 *g*. The labelled cells, mixed with 120 g of unlabelled cells, grown in the same conditions, were washed with 0.1 M NaCl and freeze-dried (yield 0.25 g of dried cells from 1 l. of culture).

Extraction and hydrolysis of lipids. Dried cells were extracted continuously (Soxhlet) for 12 hr with $CHCl_3$–MeOH (1:1). The total lipid extract (*ca* 3 g), was treated with methanolic HCl for 6 hr under reflux, and the hydrolysis mixture dried *in vacuo*.

Purification of tetraethers. Purification of the hydrolysis mixture was performed on a Si gel (Merk Kieselgel 70-230 mesh) column (40 cm, i.d. 10 mm). Elution with $CHCl_3$–Et_2O (9:1) gave glycerol-dialkyl-glycerol tetraethers (**2**) (400 mg) and $CHCl_3$–MeOH (9:1) glycerol-dialkyl-calditol tetraethers (**3**) (700 mg).

Degradation of tetraethers. The two tetraether fractions were treated with BCl_3 (5 ml in 5 ml $CHCl_3$ at 18° for 12 hr), the reaction mixtures evaporated under N_2 and then chromatographed on a Si gel column (30 cm, i.d. 10 mm). For the glycerol-dialkyl-glycerol tetraethers (**2**), $CHCl_3$ (40 ml) eluted C_{40} dichlorides (350 mg) and $CHCl_3$–MeOH (7:3, 50 ml) eluted glycerol (50 mg); the hydrolysis mixture of glycerol-dialkyl-calditol tetraethers (**3**) was resolved in the same manner, adding a further elution step with Me_2CO–MeOH–H_2O (6:2:2, 200 ml) for calditol recovery. The yield was C_{40} dichlorides (580 mg), glycerol (40 mg) and calditol (120 mg).

TLC. This was performed on 0.25 mm layers of Si gel F 254, Merck, activated by heating at 100° for 2 hr. Solvents included $CHCl_3$–MeOH–H_2O (65:25:4) for complex lipids, $CHCl_3$–Et_2O (9:1) for **2**, $CHCl_3$–MeOH (9:1) for **3**, $CHCl_3$–MeOH (7:3) for glycerol, *n*-hexane for C_{40} dichlorides and $CHCl_3$–MeOH (3:2) for calditol. Complex lipids, tetraethers and C_{40} dichlorides were detected either by exposure to I_2 vapour, or by spraying with $Ce(SO_4)_2$. Specific reagents included Dittmer and Lester [14] reagent for phospholipids. periodate–Schiff reagent [15] for *vic* glycols and diphenylamine [16] for carbohydrate-containing lipids.

Radioactivity measurements. The double labelled samples, weighed on a Cahn electrobalance, were dissolved in 5 ml of Bray's soln [17] and the radioactivity was measured on a liquid scintillation system equipped with an automatic system for selective evaluation of the absolute radioactivity from two different radionuclides. To assay the radioactivity incorporated into whole cells, 1 ml of culture was rapidly filtered on a Millipore filtration apparatus. The filter was then washed twice with 2 ml of H_2O, dried and counted for

radioactivity in the scintillation soln in the presence of Cab-O-Sil (0.1 g). In all the radioactivity measurements σ was below 0.5.

Determination of the $^3H/^{14}C$ ratio of the glycerol–glycerol phosphate pool in cells grown with double-labelled glycerol precursors. Wet cells (10 mg), grown in the presence of double labelled precursor, were frozen and suspended in 2 ml of 0.1 M Pi buffer pH 8.5; this treatment caused total cell lysis, as reported for other similar micro-organisms [18]. The soln, after addition of 0.1 mmol of glycerol and 0.1 mmol of racemic glycerol-3-phosphate, was ultrafiltered in a micro Amicon cell using a membrane to remove cell debris and high MW material. The strong alkaline hydrolysis of the freeze-dried ultrafiltrate was performed in 1 ml of 1 N NaOH at 100° for 3 hr. The neutralized hydrolysis mixture was deionized by passage through a column of AG-501 × 8D mixed bed resin and freeze-dried. The sample, dissolved in $CHCl_3$, was chromatographed on a Si gel column and eluted with $CHCl_3$–MeOH (7:3). The glycerol fraction (10 mg) dissolved in 1 ml of pyridine was treated at 0° with 0.5 ml of benzoyl chloride and the reaction mixture allowed to stand at room temp. overnight. The soln, after addition of 5 ml H_2O was then extracted twice with Et_2O; the combined Et_2O fractions were washed several times with a satd soln of $NaHCO_3$ and finally with H_2O. After solvent removal the glyceryl tribenzoate was chromatographed on a Si gel column and eluted with petrol–Et_2O (9:1). The glyceryl tribenzoate fraction was then re-crystallized from EtOH several times to constant radioactivity and the $^3H/^{14}C$ ratio determined.

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