# BIOSYNTHESIS OF GLUCURONOSYL DIGLYCERIDE BY A CELL-FREE SYSTEM OBTAINED FROM A MODERATELY HALOPHILIC-HALOTOLERANT BACTERIUM

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## 1. Introduction

Glucuronosyl diglyceride was recently isolated by Wilkinson from several species of the genus Pseudomonas and its structure was determined [1, 2]. In a previous communication [3] we described the lipid composition of a moderately halophilic-halotolerant gram negative rod. These bacteria were found to contain a glucuronosyl diglyceride derivative which was different from that described by Wilkinson. When these bacteria were grown on a synthetic, high salt medium with glucose as sole carbon source, this lipid was the major glycolipid present; it amounted to 28% of the total polar lipids. In addition, small amounts of another glucuronic acid-containing glycolipid were found. This compound seems identical to the glucuronosyl diglyceride of Wilkinson. From these bacteria, a cell-free preparation was obtained by sonication, which incorporated $^{14}$C-labelled glucuronic acid added as UDP-glucuronic acid, and $^{14}$C-labelled diglycerides into polar lipids. The properties of this system and the identification of the lipid formed will be described in this communication.

## 2. Materials and methods

### 2.1. *Organism and growth conditions*

The source and properties of the bacteria used were previously described [3]. The organisms were grown for 48 hr at 37° in a liquid medium which contained (g per l): $NH_4Cl$ 5, $NH_4NO_3$ 1, $Na_2SO_4$ 2, $K_2HPO_4$ 3, $KH_2PO_4$ 1, $CaCl_2$ 0.1, NaCl 87.7 (1.5 M), KCl 37 (0.5 M), $MgCl_2$ 3 (0.05 M) and glucose 0.5 g at pH 6. After harvesting, the cells were washed twice with a solution which contained NaCl 2 M and $MgCl_2$ 0.05 M.

### 2.2. *Preparation of cell-free particles*

1 g of washed cells were suspended in 2 ml of Tris buffer 0.1 M, pH 7.4 and subjected to intermitent sonication (5 times 1 min with 30 sec intervals). To this suspension DNase (10 $\mu$g per ml) was added. After a few min in the cold, the mixture was centrifuged at 15,000 *g* for 10 min to remove unbroken cells and debris. From the supernatant, a particulate fraction was precipitated at 140,000 *g* for 45 min. The pellet obtained was suspended in a small volume of Tris buffer, 0.1 M, pH 7.4 and the protein concentration of the suspension adjusted to 20 mg per ml. This suspension was divided into 4 mg batches and rapidly lyophilized.

### 2.3. *Assay of glucuronosyldiglyceride synthesis*

To the lyophilized particles 0.1 $\mu$mole of $^{14}$C-labelled-UDP glucuronic acid containing 150,000 cpm, $MgCl_2$ 5 mM and Tris buffer pH 8.0, 50 mM in a total volume of 0.9 ml were added. Whenever diglycerides were also added, they were dissolved in a small volume of benzene and added directly onto the lyophilized particles [4]. The benzene was removed with a gentle stream of nitrogen and the rest of the reaction mixture was then added. Incubation was generally at 37° for 1 hr.

2.4. *Isolation and identification of lipid synthesized*

The reaction was stopped by adding 4 ml of chloroform–methanol (1:2, v/v) and the lipids were extracted according to the method of Bligh and Dyer [5]. To identify the product formed and to determine the amount of radioactivity incorporated into glucuronic acid-containing glycolipids a 2 step thin layer chromatographic (TLC) system was adapted. Known aliquots were applied at the origin; the plates were first developed with acetone–petroleum ether (B. P. 40°–60°) (1:3, v/v) to remove neutral lipids [6]. The plates were dried and developed in the same direction with one of the following solvents: A = chloroform–methanol–acetic acid–water, 100:20:12:5, by vol. [3] or B = 85:15:10:4, by vol. [1]. Spots were visualized with $I_2$ vapor and identified by specific spray reagents as previously described [3]. For radioactive assays the spots were scraped off the plates and counted as previously described [7].

2.5. *Preparation of diglycerides*

Lipids were extracted from cells grown on synthetic medium and were purified on a silicic acid column. The phospholipids were dissolved in ether and incubated with Phospholipase C of *Bacillus cereus* for 2.5 hr [8]. The reaction was stopped by the addition of chloroform–methanol and the lipids were extracted as previously described. To separate the diglycerides from the remaining polar lipids, the chloroform extract was applied onto a silicic acid column. Diglycerides were eluted with chloroform. The purity of the preparation was checked by TLC [9]. The amount of diglyceride formed was determined by the hydroxamic acid method [10]. $^{14}$C-labelled-diglycerides were obtained by the same method, from cells which were grown in U-$^{14}$C-labelled glucose. In one experiment, $^{14}$C-labelled diglycerides were formed by incubating locust fat-body microsomes and phosphatidate phosphatase with 1-$^{14}$C-labelled palmitic or oleic acid [7].

Methods used for the identification and quantitative estimation of fatty acids, phosphate and uronic acid were previously described [3].

2.6. *Materials*

DNase was purchased from Worthington Biochemical Corporation, New Jersey, Phospholipase C of *B. cereus* from Makor Chemical Ltd., Jerusalem. Glucuronic acid, UDP-glucuronic acid and UDP-glucose from Boehringer, Mannheim. All $^{14}$C-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks., England. The purity of the $^{14}$C-labelled UDP-glucuronic acid was checked by TLC on cellulose MN-300 coated plates employing n-propanol – conc. ammonia – water (60:30:10 + 2g EDTA/l) as developing solvent. The amount of radioactivity added was calculated from the amount associated with the UDP-glucuronic acid spot.

## 3. Results

3.1. *Lipid composition of cells grown on glucose*

The lipid composition of bacteria grown on nutrient broth and on glucose as sole carbon source, was compared by TLC. In cells grown on glucose, the lipid previously identified as a glucuronosyl diglyceride derivative was the major glycolipid present, while glucosyl phosphatidyl-glycerol was found in relatively small amounts. In addition, small amounts of another glucuronic acid-containing lipid were detected. On silica gel plates employing solvent B, this glycolipid migrated between cardiolipin and phosphatidylethanolamine ($R_f$ = 0.67) like the glucuronosyl diglyceride which was isolated by Wilkinson and which he kindly sent us. This compound moved faster than the major glucuronic acid containing compound which had an $R_f$ of 0.17. Quantitative determinations showed that the fast glucuronosyl diglyceride amounted to approximately 1% of the total polar lipids.

The unusual high content of cyclopropane acids was also found in the lipids isolated from bacteria grown on glucose.

3.2. *Incorporation of $^{14}$C-glucuronic acid into glycolipids*

From cells grown on glucose a cell-free extract was obtained by sonication. When this extract was incubated with UDP-$^{14}$C-glucuronate, $^{14}$C-glucuronate was incorporated into glycolipids. This extract was fractionated by centrifugation. 95% of the activity was recovered in the 140,000 *g* particles. Addition of the soluble supernatant to these particles had no effect. The particles could not utilize $^{14}$C-glucuronic acid. Addition of "cold" glucuronic acid in the pre-

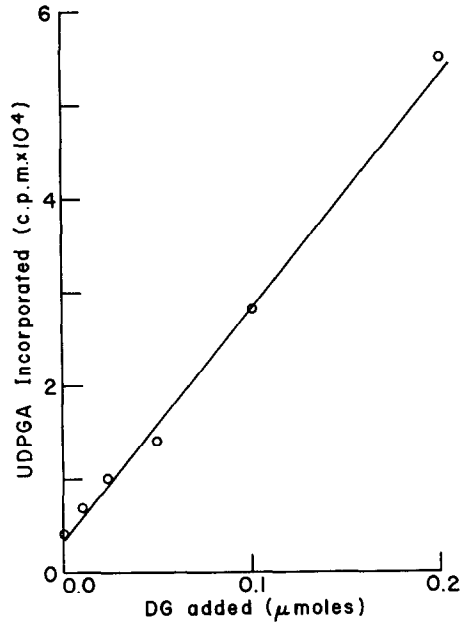


Fig. 1. Effect of diglycerides on the incorporation of UDP-$^{14}$C-glucuronate into glycolipids. 4 mg of lyophilized particles were incubated for 1 hr in Tris buffer pH 8.0 with 0.1 μmoles of UDP-$^{14}$C-glucuronate (150,000 cpm). Different amounts of DG in benzene were directly adsorbed into the particles.

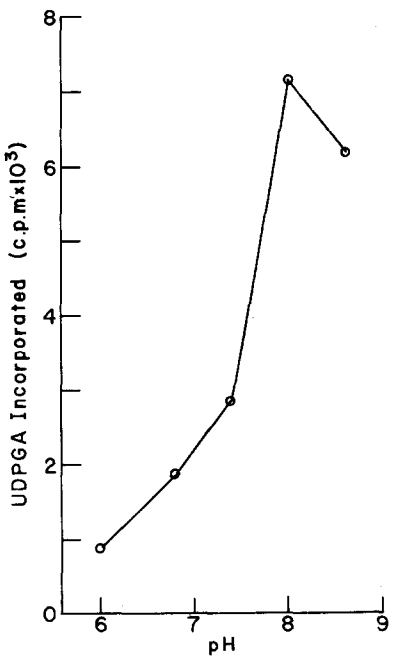


Fig. 2. pH Dependence of UDP-$^{14}$C-glucuronate incorporation. 4 mg of lyophilized particles were incubated for 1 hr in Tris-maleate buffer, with 0.1 μmoles UDP-$^{14}$C-glucuronate (150,000 cpm).

sense of UDP-$^{14}$C-glucuronate did not inhibit the incorporation of the latter.

### 3.3. *Effect of diglycerides and identification of the lipid synthesized*

The incorporation of $^{14}$C-glucuronate was stimulated 10-fold by the addition of 0.1 μmole of bacterial 1, 2-diglycerides. The response to the addition of diglycerides was linear; even in the presence of 0.2 μmoles no inhibition was noted (fig. 1).

To identify the $^{14}$C-labelled compound synthesized, the lipid mixture was separated by TLC as described in the Methods. Only 1 labelled spot was found. In solvent B the label coincided with the fast glucuronic acid-containing glycolipid. No counts were found in the major glucuronic acid-containing lipid. When the labelled lipid was recovered and subjected to acid hydrolysis, all the radioactivity was recovered in glucuronic acid.

When particles were incubated with "cold" UDP-glucuronate and $^{14}$C-labelled bacterial diglycerides, radioactivity was found only in the fast glucuronosyl diglyceride. The incorporation of $^{14}$C-diglycerides was absolutely dependent on the addition of UDP-glucuronate.

In parallel experiments, approx. equal amounts of $^{14}$C-glucuronate and $^{14}$C-diglycerides were incorporated into glucuronosyldiglyceride (table 1).

To test the specificity of the system, 1, 2-diglycerides enriched with $^{14}$C-palmitate or $^{14}$C-oleate were

Table 1
Incorporation of UDP-$^{14}$C-glucuronate and $^{14}$C-diglyceride into glucuronosyl diglyceride.

| Compounds added | $^{14}$C-incorporated | |
|---|---|---|
| | cpm | nmoles |
| UDPGA-$^{14}$C (500,000 cpm) + DG | 176,000 | 35 |
| UDPGA + DG-$^{14}$C (3,500 cpm) | 1,160 | 33 |

4 mg lyophilized particles were incubated for 1hr in Tris buffers, pH 8.0 with 0.1 μmole of each: UDP-glucuronate (UDPGA) and 1, 2-diglyceride (DG).

isolated from locust fat body microsomes. These diglycerides were not utilized by the bacterial system.

### 3.4. *Properties of the system*

Employing Tris buffer, the system showed maximal activity at pH 8.0. Addition of 5 mM $MgCl_2$ caused a 2-fold stimulation. Since the particles were isolated from bacteria which were grown in a high salt medium, NaCl and KCl were added. The addition of salt was inhibitory at all concentrations tested.

Under optimal conditions, the incorporation of $^{14}$C-glucuronate increased linearly when the amount of particles added or the time of incubation were increased.

## 4. Summary and discussion

It has been shown in this paper that cell-free particles obtained from moderately halophilic-halotolerant bacteria catalyzed the incorporation of equal amounts of $^{14}$C-labelled glucuronic acid added as UDP-$^{14}$C-glucuronate and $^{14}$C-labelled diglycerides into a compound identified as glucuronosyl diglyceride by comparison with a similar compound isolated by Wilkinson from *Ps. rubescens* and *Ps. diminuta*. The major glucuronic acid-containing glycolipid which in the intact bacteria amounted to 28% of the total polar lipids did not contain radioactivity under the experimental conditions tested. The system required the addition of 1, 2-diglycerides which contained cyclopropane fatty acyl groups like those found in the phospho- and glycolipids of the bacteria. Addition of $MgCl_2$ was stimulatory. Thus the biosynthesis of glucuronosyl diglyceride, like that of mannosyl- and glucosyl diglycerides in bacteria [11, 4, 12] and galactosyl diglyceride in plants [13], proceeds by the transfer of glycosyl residues from a donor glycosyl dinucleotide to an acceptor 1, 2-diglyceride.

Although the particles were isolated from moderately halophilic bacteria, the addition of salt to the reaction mixture markedly inhibited the reaction. These results are different from those reported for several membrane bound enzymes from extreme halophiles [14] which required salt for activity.

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