A method for the specific labeling of the glycerol in glyceride-containing lipids of *Streptococcus faecalis* ATCC 9790

RONALD A. PIERINGER[1] AND RICHARD T. AMBRON[2]

Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Summary Cells of *Streptococcus faecalis* incorporate radioactive glycerol exclusively into the glycerol moieties of monoglucosyl diglyceride, diglucosyl diglyceride, phosphatidyl diglucosyl diglyceride, phosphatidylglycerol, diphosphatidylglycerol, phosphatidic acid, diglyceride, and a phospholipid tentatively identified as an amino acyl phosphatidylglycerol. Phosphatidylglycerol is one of the major radioactive lipids synthesized and contains an equal amount of radioactivity in each of its two glycerol moieties.

Supplementary key words glycerol-labeled lipids of *S. faecalis* · [^{14}C]phosphatidyl glucosyl diglyceride · [^{14}C]diphosphatidylglycerol · [^{14}C]glucosyl diglycerides

WHILE carrying out experiments on the elucidation of the structure of phosphatidyl diglucosyl diglyceride of *Streptococcus faecalis* ATCC 9790, Ambron and Pieringer (1) found that dividing cells of *S. faecalis* incorporated [^{14}C]glycerol exclusively into the glycerol moieties of the phosphoglucolipid. This specificity of labeling made the characterization of the phosphoglucolipid significantly easier. Because of the further potential value of this technique, we extended our investigations on the specificity of glycerol labeling to phosphatidylglycerol and the other lipids of *S. faecalis*. The results of these studies are reported here.

Methods and results. *S. faecalis* ATCC 9790 was maintained on 3% trypticase soy agar slants. Liquid cultures of *S. faecalis* were made using the complete (lipid-free) basal medium of Shockman (2), which can be purchased from General Biochemicals, Chagrin Falls, Ohio, as "synthetic basal medium for *S. faecalis* (Dr. Shockman's formulation)." Because of possible instability to heat, the medium was sterilized by filtration (Millipore) rather than by autoclaving. Radioactive lipids were produced by adding 50 μCi of 1- or 3-^{14}C-labeled glycerol (27 mCi/mmole, Amersham/Searle, Arlington Heights, Ill.) and a 5-ml inoculum of *S. faecalis*, freshly grown in basal medium, to 500 ml of freshly prepared basal medium contained in a 1-l culture flask. The cells were grown into log phase (to an unadjusted optical density of about 1.1 at 675 nm) at 37°C with gentle shaking (little if any aeration except at the surface) and were harvested by centrifugation. The ^{14}C-labeled lipids were extracted by mixing the pelleted cells with 100 ml of chloroform–methanol 2:1 (v/v) overnight at room temperature. After this mixture was washed several times with 50-ml portions of water, the chloroform phase was adjusted to a known volume and the ^{14}C content was determined with a gas-flow Geiger counter having an efficiency of about 25%. Approximately 12 × 10^6 dpm (or 10.8% of the starting dpm of [^{14}C]glycerol) of ^{14}C-labeled lipids were obtained by this procedure in each of the several experiments carried out. The radioactive lipids in the crude mixture were analyzed qualitatively and quantitatively by a series of chromatographic and electrophoretic techniques previously described (1). A partial separation of the ^{14}C-labeled lipids was achieved by chromatographing a portion of the total mixture on SG-81 paper (H. Reeve Angel and Co., Clifton, N.J.) developed overnight with diisobutyl ketone–acetic acid–water 40:25:5 (v/v/v) (3) (system A). A strip scan of the chromatogram revealed five major radioactive peaks at (I) 5.6, (II) 10, (III) 16.8, (IV) 24.1, and (V) 30.4 cm from the origin. Each of these peaks was eluted with chloroform–methanol 1:1 (v/v) and deacylated by the procedure described below. The deacylated products were purified by one of the following systems: (system B) electrophoresis on Schleicher and Schuell (Keene, N.H.) no. 2040-B paper buffered with pyridine–acetic acid–water 1:10:89 (v/v/v), pH 3.9, at 50 V/cm for 75 min; and (system C) chromatography on Whatman no. 1 paper developed with *n*-butanol–

[1] Recipient of Research Career Development Award NS-12776. To whom inquiries are to be made.

[2] Present address: Department of Neurobiology and Behavior, Public Health Research Institute, New York 10016.

pyridine–water 6:4:3 (v/v/v). The amount of ^{14}C in each of these purified compounds was measured in a gas-flow Geiger counter. The following data report the identity of the lipid, the distance the deacylated derivative of the lipid traveled in systems B or C, and the percentage of the radioactivity of the total lipid extract found in the purified lipid: phosphatidyl diglucosyl diglyceride, 7.5 cm in B, 2.4%; diphosphatidylglycerol, 27 cm in B, 9.7%; phosphatidic acid, 19 cm in B, 1.2%; diglyceride, 14.3 cm in C, 3.2%; monoglucosyl diglyceride, 9.3 cm in C, 2%; diglucosyl diglyceride, 5.3 cm in C, 17.3%; phosphatidylglycerol, 14.9 cm in B, 25.8%; and amino acyl phosphatidylglycerol (tentative identification), 14.9 cm in B, 38.4%. It should be noted that both phosphatidylglycerol and its aminoacyl derivative yield the same deacylation product (glycerylphosphorylglycerol). Although ^{14}C-labeled phosphatidylglycerol was shown by cochromatography experiments to exist in peak III of system A, the exact location of the aminoacyl derivative could not be proven because standard amino acyl phosphatidylglycerol was not available. However, this phospholipid does occur in *S. faecalis* (4), and because of its greater polarity it would run slower in system A (at peak II instead of III) than phosphatidylglycerol (4). Thus, we are tentatively identifying the ^{14}C-labeled phospholipid at peak II of system A that yielded glyceryl phosphoryl glycerol on deacylation as an amino acyl phosphatidylglycerol.

Deacylation of a portion of the total ^{14}C-labeled lipid mixture was carried out in a solution of 1 ml of toluene, 1 ml of methanol, and 2 ml of 0.2 M methanolic KOH heated to 37°C for 15 min (5). When only the water-soluble products of the hydrolysis were to be measured, 2 ml each of $CHCl_3$ and water was then added to the solution. The water phase was passed over protonated Dowex 50 and then neutralized with NH_4OH. However, our first interest was to measure the amount of ^{14}C in the fatty acids released by the alkaline methanolysis. This was accomplished by adding 2 ml of $CHCl_3$ and 2 ml of 1 N HCl to the solution containing the deacylated derivatives of the total lipid mixture. After mixing, each of the resulting two phases was measured for ^{14}C content. The results of a typical experiment were the following: in the water phase there was 25,650 cpm; in the $CHCl_3$ phase containing the released fatty acids and any unreacted lipids there was 60 cpm. In a similar experiment starting with more than 200,000 cpm of a mixture of ^{14}C-labeled lipids, no counts above background were detected in the $CHCl_3$ phase. These results demonstrate that the radioactivity of [^{14}C]glycerol was not incorporated into the fatty acid moieties of any of the lipids of *S. faecalis* ATCC 9790.

When the water-soluble radioactive compounds derived from the deacylated lipids were treated with 1 N HCl at 100°C for several hours, the only detectable radioactive compounds were glycerol and glycerophosphate. The latter compounds were identified by chromatography with standards in the following three systems: system B, in which glycerophosphate travels 19 cm and glycerol remains at the origin; system C, in which glycerophosphate remains at the origin and the R_F of glycerol is 0.62; and system D, consisting of Whatman no. 1 paper developed in 1 M ammonium acetate (pH 7.5)–ethanol 7:3 (v/v), in which the R_F values of glycerophosphate and glycerol are 0.84 and 0.93, respectively. As in our previous study (1), no radioactive glucose could be detected. Thus, the radioactive label from [^{14}C]glycerol is incorporated exclusively into the glycerol moieties of the glyceride lipids of *S. faecalis*.

Because phosphatidylglycerol contains a significant amount of the radioactivity taken up by the cells into lipid and is probably the most ubiquitous phospholipid found in nature, it should be more useful than perhaps any of the other labeled lipids considered in this study. Therefore, the degree and the pattern of the glycerol labeling in phosphatidylgylcerol were examined in greater detail. After purification by silicic acid column chromatography (4), a dried sample of the [^{14}C]glycerol-labeled phosphatidylglycerol was treated with 1 ml of 90% acetic acid at 100°C for 20 min in a sealed tube. Under these conditions of hydrolysis, phosphatidylglycerol is specifically degraded to diglyceride and glycerophosphate (plus a small amount of acetyl glycerophosphate) (6, 7). Carrier nonradioactive dipalmitin (0.05 μmole) and glycerophosphate (0.4 μmole) were added and the solution was dried under a stream of nitrogen. The residue was dissolved in a mixture of 1 ml of methanol, 2 ml of chloroform, and 2 ml of water. After mixing, the radioactivity in the aqueous and chloroform phases was determined and was found to be 7248 and 7448 cpm, respectively. On chromatography, the water phase yielded only glycerophosphate (and a small amount of acetyl glycerophosphate) as determined in system B, and the chloroform phase contained only diglyceride plus a trace amount of unreacted phosphatidylglycerol as determined by chromatography system A. These results indicate that the glycerol moieties of phosphatidylglycerol are equally labeled under the growth conditions used.

The specific radioactivity of the [^{14}C]glycerol moieties of the phosphatidylglycerol was also determined. Radiochemically pure phosphatidylglycerol was deacylated, and the water-soluble radioactive products were treated with 2 N HCl for 17 hr at 100–103°C. As already shown above, the radioactive products of this reaction are glycerophosphate and glycerol. After removal of the HCl by evaporation, the products were dissolved in water and were separated by anion exchange (Dowex 2, CO_3^{2-}) column chromatography. Only [^{14}C]glycerol passes through the column under these conditions. An aliquot

of the eluant was chromatographed in system C. Only one radioactive compound was present, and this ran exactly with standard glycerol. Other aliquots were taken for measuring radioactivity and glycerol concentration (assayed by periodate oxidation) (8). The specific activity of the [^{14}C]glycerol was 70 cpm/nmole as determined by a Geiger counter having an approximate efficiency of 25%. The specific activity of the phosphatidylglycerol would of course be 140 cpm/nmole.

Discussion. The technique described in this paper provides a reasonably easy method for preparing seven lipids labeled specifically in the glycerol moieties. To our knowledge none of these lipids labeled with [^{14}C]-glycerol are available from commercial sources. Of the labeled lipids considered in this study, phosphatidylglycerol is perhaps of greatest value because its wider distribution in nature suggests that it has the potential to function as a substrate (or cofactor) in many more enzyme systems from different sources than would the other lipids. Also, it is one of the most heavily labeled lipids made by *S. faecalis*. In our experience, the location of the label in the glycerol moiety, rather than in some other group, of the lipid substrate often is of some advantage. For example, in biosynthetic studies using crude enzyme preparations, lipid substrates labeled in the fatty acid group many times produce unwanted radioactive free fatty acids as side-products; these can be relatively difficult to separate from the primary product of the reaction.

One disadvantage of the technique may be that the labeled lipids will have a fatty acid composition characteristic of *S. faecalis*. The fatty acid content of the lipids of *S. faecalis* may not be suitable or natural to a given system under study. The fatty acid composition of the total lipids of *S. faecalis* (relative to $C_{16:0}$ set at 1.00) has been reported by Toon, Brown, and Baddiley (9) to be $C_{16:0}$ (1.00), $C_{18:1}$ (0.95), C_{19}-cyclopropane (0.4), $C_{16:1}$ (0.20), and $C_{14:0}$ (0.09). This composition resembles that derived from the total lipids of *S. cremonis* and *S. lactis* var. *maltigens* (10).

Shockman[3] has observed that *S. faecalis* ATCC 9790 does not grow when glycerol is substituted for glucose in the chemically defined medium used in our incubations. Undoubtedly, this observation must be directly related to our finding that [^{14}C]glycerol is incorporated intact and is found only in the glycerol moieties of lipids. The reason for the lack of growth on glycerol, that is, the inability to use glycerol as an energy source, is unknown and is open to conjecture. Two speculative points can be made: first, in metabolizing glycerol to lactate, the *S. faecalis* 9790 under our growth conditions (chemically defined medium) has the potential of creating an excess of reducing equivalents in that there are two reactions that reduce NAD and only one that oxidizes NADH; and second, there is the possibility that if some radioactivity from glycerol reached the acetate pool (precursor of fatty lipids) it would be diluted extensively by exogenous acetate present in the medium. It is unlikely that the [^{14}C]glycerol labeling pattern observed with *S. faecalis* ATCC 9790 will be observed with many other strains of *S. faecalis* because other strains were observed to grow on glycerol aerobically as well as anaerobically (11). However, *S. faecium* did not grow on glycerol anaerobically (11). Thus, in at least this one respect, *S. faecalis* ATCC 9790 appears to resemble *S. faecium* rather than *S. faecalis*.

This investigation was supported by research grants AI-05730 and 1-P01-HD-05874 from the U.S. Public Health Service and GB 24802 from the National Science Foundation.

We are grateful to Miss Segunda Lim and Miss Erlinda Cabacungan for contributing expert technical assistance to various phases of this study.

Manuscript received 21 August 1972; accepted 29 January 1973.

[3] Shockman, G. D. Personal communication.

REFERENCES

1. Ambron, R. T., and R. A. Pieringer. 1971. The metabolism of glyceride glycolipids. V. Identification of the membrane lipid formed from diglucosyl diglyceride in *Streptococcus faecalis* ATCC 9790 as an acylated derivative of glyceryl phosphoryl diglucosyl glycerol. *J. Biol. Chem.* **246:** 4216–4225.
2. Shockman, G. D. 1963. Amino acids. *In* Analytical Microbiology. F. Kavanagh, editor. Academic Press, New York. 644–673. (Table XII, 644–645.)
3. Kates, M. 1967. Paper chromatography of phosphatides and glycolipids on silicic-acid-impregnated filter paper. *In* Lipid Chromatographic Analysis. Vol. 1. G. V. Marinetti, editor. Marcel Dekker, New York. 1–39.
4. Vorbeck, M. L., and G. V. Marinetti. 1965. Intracellular distribution and characterization of the lipids of *Streptococcus faecalis* (ATCC 9790). *Biochemistry.* **4:** 296–305.
5. Steiner, S., S. F. Conti, and R. L. Lester. 1969. Separation and identification of the polar lipids of *Chromatium* strain D. *J. Bacteriol.* **98:** 10–15.
6. Kiyasu, J. Y., R. A. Pieringer, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238:** 2293–2298.
7. Coulon-Morelec, M. J., M. Faure, and J. Maréchal. 1960. Étude du mécanisme de la libération des diglycérides des phosphatides sous l'action de l'acide acétique chaud. *Bull. Soc. Chim. Biol.* **42:** 867–876.
8. Frisell, W. R., L. A. Meech, and C. G. Mackenzie. 1954. A simplified photometric analysis for serine and formaldehyde. *J. Biol. Chem.* **207:** 709–716.
9. Toon, P., P. E. Brown, and J. Baddiley. 1972. The lipid–teichoic acid complex in the cytoplasmic membrane of *Streptococcus faecalis* N.C.I.B. 8191. *Biochem. J.* **127:** 399–409.
10. Kates, M. 1964. Bacterial lipids. *Adv. Lipid Res.* **2:** 17–90.
11. Deibel, R. H. 1964. The group D streptococci. *Bacteriol. Rev.* **28:** 330–366.