USE OF [1 or 3-^{3}H, U-^{14}C]GLUCOSE TO ESTIMATE THE SYNTHESIS OF GLYCEROLIPIDS VIA ACYL DIHYDROXYACETONE PHOSPHATE

Robert J. Pollock, Amiya K. Hajra, William R. Folk, and Bernard W. Agranoff

Mental Health Research Institute and Department of Biological Chemistry
The University of Michigan, Ann Arbor, Michigan 48104

Received May 19,1975

Summary: BHK-21-cl3 fibroblasts were incubated with [1-^{3}H, U-^{14}C]glucose or [3-^{3}H, U-^{14}C]glucose to produce intracellular [^{3}H]NADPH *via* the phosphogluconate pathway. ^{3}H and ^{14}C were then determined at the three positions of glycerol in glycerol phosphate, saponifiable glycerolipids, alkyl ether glycerolipids and plasmalogens. The ^{3}H/^{14}C ratio at C-2 in glycerol of saponifiable glycerolipids is 2-10 fold greater than in glycerol phosphate and approaches the ratio found in ether-containing glycerolipids. This suggests that a significant fraction of the glycerolipids in BHK-21-cl3 fibroblasts is synthesized *via* acyl dihydroxyacetone phosphate.

There are at least two pathways through which lipid glycerol can be synthesized from glycolytic intermediates - the glycerol phosphate (GP)[1] and acyl dihydroxyacetone phosphate (DHAP)[1] pathways (1-3). The GP pathway preferentially consumes NADH (4) whereas the acyl DHAP pathway uses NADPH (5). This difference has previously been used to distinguish glycerolipid synthesis *via* the two pathways in disrupted cell preparations (1,2) but not as yet in intact cells. We report here the use of [1-^{3}H, U-^{14}C]glucose and [3-^{3}H, U-^{14}C] glucose to estimate the relative importance of the two pathways in intact BHK-21-cl3 fibroblasts. Both [1-^{3}H]glucose and [3-^{3}H]glucose are expected to provide intracellular [^{3}H]NADPH *via* the phosphogluconate oxidative pathway and both have been previously used to measure NADPH metabolism within cells (6-9). Glycerolipid synthesized *via* acyl DHAP should contain ^{3}H at C-2 of glycerol whereas that synthesized *via* GP should not. Ether-containing glycerolipids are thought to be synthesized only *via* acyl DHAP, so NADPH is an obligatory

[1] Abbreviations: GP, *sn* glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate.

intermediate (2,5). They have therefore been used to estimate the result expected from complete glycerolipid synthesis via the acyl DHAP pathway.

MATERIALS AND METHODS

BHK-21-c13 cells were grown at 37° in MEM medium (AutoPow, Flow Labs) supplemented with 5% tryptose phosphate broth and 10% calf serum. The cells were harvested by trypsinization and washed twice with Krebs-Ringer phosphate buffer, pH 7.4. [U-^{14}C]Glucose, [1-^{3}H]glucose and [3-^{3}H]glucose were used as purchased from New England Nuclear Corporation. Sodium bis(2-methoxyethoxy)-aluminum hydride (Red-al) was from Aldrich Chemical Company, and Sigma Chemical Company was the source of glycerokinase (EC 2.7.1.30), alkaline phosphatase (EC 3.1.3.1), and Folch fraction V lipid extract of bovine brain (rich in ether-containing phospholipids).

BHK-21-c13 cells were incubated at 38° with shaking in glass scintillation-counting vials containing 2 ml of the Krebs-Ringer phosphate buffer. After 10 min, 0.1 ml of 21 mM [1-^{3}H, U-^{14}C]glucose or [3-^{3}H, U-^{14}C]glucose, containing 100 µCi ^{3}H and 20 µCi ^{14}C, was added. The reaction was stopped after an additional 30 min by transferring the cell suspension to 7.9 ml of chloroform-methanol 1:2 (by vol) then sonicating. Protein in the centrifuged pellet was measured (10). The supernatant solution was partitioned by mixing with 2.6 ml of chloroform plus 2.6 ml of water (11).

GP was isolated from the aqueous phase as the barium salt (12), converted to the free acid with Dowex 50W-X8 (H^+), then subjected to high voltage paper electrophoresis, first at pH 4.3 with 1.7 M pyridinium acetate, then at pH 1.5 with 60 mM sodium oxalate (13). This preparation was then dephosphorylated with alkaline phosphatase, deionized, rephosphorylated with glycerokinase and reisolated as the barium salt. The glycerol moieties of the glycerolipids were purified from the organic phase of the partition. Five mg of carrier Folch fraction V brain lipids were added, the lipid extract was washed by re-extraction (14), and then hydrogenolysed by Red-al (15,16). The glycerol moiety of saponifiable glycerolipids was purified from the aqueous phase of the hydrogeno-

lysis product by deionization and conversion by glycerokinase to glycerol phosphate. The GP product was isolated as the barium salt. Alkyl glycerols and 1'-alkenyl glycerols were isolated from an ether extract of the hydrogenolysis product by thin layer chromatography (16). The glycerol moiety of the 1'-alkenyl glycerols was obtained by hydrolysis (17) followed by deionization. Glycerol from alkyl glycerols was obtained by cleaving the ether bond with hydriodic acid (18). Five mg of batyl alcohol were added and the dried ether lipid suspended in 2 ml of 47% aqueous hydriodic acid. After heating for 2.5 hr at 105° in a sealed tube, the lipid products were extracted by chloroform and the aqueous phase evaporated to remove hydriodic acid. After deionization, glycerol was purified by enzymatic conversion to GP.

The distribution of ^{3}H and ^{14}C in glycerol was determined after periodic acid degradation. In order to distinguish radioactivity at C-1 from that at C-3, half the glycerol moiety was degraded as free glycerol and half as GP (1). Since radioactivity in glycerol from plasmalogens was low, only free glycerol was degraded. Radioactivity was measured by liquid scintillation counting using either an external standard or internal standards to estimate quenching.

RESULTS

BHK-21-c13 fibroblasts incorporated into lipid about 2.0, 1.3, and 0.9 nmoles of [U-^{14}C]glucose, [1-^{3}H]glucose, and [3-^{3}H]glucose, respectively, per mg protein. Of this radioactivity, roughly 40% was in saponifiable lipid glycerol and 20% in fatty acids. The glycerol moiety of the alkyl ethers contains about ten fold more radioactivity than does that of the plasmalogens (Table 1) even though BHK-21-Wi2 cells reportedly contain similar amounts of these two types of ether lipids in the phospholipid fraction (19,20).

The ^{3}H and ^{14}C at various positions within the glycerol of GP, saponifiable glycerolipids, alkyl ethers and plasmalogens is largely that anticipated. The distribution of ^{14}C is nearly uniform, although C-1 often shows somewhat less and C-3 somewhat more than the expected amount of radioactivity. Most of the ^{3}H apparently enters *via* glycolysis so that [1-^{3}H]glucose labels C-3 of the

TABLE I. Distribution of Radioactivity in Glycerol Moieties from BHK-21-c13 Cells Incubated with [1 or 3-^{3}H,U-^{14}C]Glucose

Glucose Substrate	Source of Glycerol	Radioactivity (dpm)	Distribution of Label (%) C-1	C-2	C-3	C-1+C-3
1-^{3}H,U-^{14}C	Glycerol phosphate	12,400	4.4	2.0	94	98
		3,000	30	35	35	65
	Saponifiable lipids	83,300	1.2	4.1	95	96
		20,000	33	32	35	68
	Alkyl ether lipids	4,960	0.9	5.3	94	95
		1,350	26	33	41	67
	Plasmalogens	860	-	6.3	-	94
		180	-	28	-	72
3-^{3}H,U-^{14}C	Glycerol phosphate	15,700	86	1.7	12	98
		7,810	30	33	37	67
	Saponifiable lipids	72,800	73	12	15	89
		26,600	29	33	38	67
	Alkyl ether lipids	1,020	0.2	90	10	10
		3,100	24	33	43	67
	Plasmalogens	100	-	94	-	6
		190	-	37	-	63

Cell suspensions were incubated at 38° in 2 ml of Krebs-Ringer phosphate buffer (pH 7.4) on a metabolic shaker under an air atmosphere. After 10 min, 0.1 ml of 21 mM D-glucose, labeled as indicated, was added. The incubation was then continued 30 min more before being stopped by the Bligh-Dyer (11) extraction of lipid. Cell protein was 2.9 and 3.8 mg in the incubations with [1-^{3}H,U-^{14}C]glucose and [3-^{3}H,U-^{14}C]glucose, respectively. The lipid extract was hydrogenolysed with bis(2-methoxyethoxy)-aluminum hydride (15,16) to produce saponifiable lipid glycerol. Ether-containing glycerolipids were purified from this hydrogenolysate and the glycerol in alkyl ether lipids and plasmalogens isolated by hydriodic acid cleavage (18) and acid hydrolysis (17), respectively. Glycerol phosphate was purified from the aqueous phase of the Bligh-Dyer extract. Except for plasmalogen glycerol, the glycerol moieties were degraded with periodate before and after enzymatic conversion to glycerol phosphate to determine the radioactivity at each position (1). For each glycerol moiety, values for ^{3}H are presented in the first line and ^{14}C in the second.

glycerol while [3-^{3}H]glucose labels C-1 (8). An interesting exception to this distribution pattern is the absence of ^{3}H at C-1 of glycerol in ether glycerolipids formed from [3-^{3}H]glucose, indicating that GP is not a direct precursor

of ether-containing glycerolipids thus supporting the view that they are synthesized via acyl DHAP (3). In subcellular fractions, this C-1 hydrogen has been found to exchange with water during conversion of acyl DHAP to alkyl DHAP (21) and the data here support the occurrence of this exchange in intact cells. A similar exchange of the C-1 hydrogen can occur during the DHAP to glyceraldehyde-3-phosphate equilibrium catalyzed by triose phosphate isomerase (EC 5.3.1.1). The appearance of ^{3}H in C-1 of GP and saponifiable glycerolipids indicates this latter exchange is incomplete, as has been previously reported for adipose tissue (6).

Ratios of $^{3}H/^{14}C$ at C-2 of the four glycerol moieties (Table 2) show that the glycerol entering saponifiable glycerolipids contains relatively more ^{3}H at this position than does the putative precursor, GP, and its ratio approaches that seen in glycerol of ether lipids.

TABLE II. $^{3}H/^{14}C$ Ratios at Position 2 of Glycerol Moieties from BHK-21-cl3 Cells Incubated with [1 or 3-^{3}H,U-^{14}C]Glucose

Glucose Substrate	Source of Glycerol	2-^{3}H/2-^{14}C
1-^{3}H,U-^{14}C	Glycerol phosphate	0.23
	Saponifiable Lipids	0.54
	Alkyl ether Lipids	0.57
	Plasmalogens	0.98
3-^{3}H,U-^{14}C	Glycerol phosphate	0.08
	Saponifiable Lipids	0.89
	Alkyl ether Lipids	0.89
	Plasmalogens	1.47

DISCUSSION

When cells are incubated with [1 or 3-^{3}H, U-^{14}C]glucose, ^{3}H at C-2 of the glycerol in both saponifiable and ether-containing glycerolipids is expected to enter only from NADPH since we assume that the [^{3}H]glucose used will not give rise to labeled NADH. Since there is a considerably greater isotope effect in the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) step than in the 6-phosphogluconate dehydrogenase (EC 1.1.1.44) step, [3-^{3}H]glucose should be the better precursor (8). ^{14}C is presumed to enter this position in ether-containing glycerolipids *via* acyl DHAP only (3) but will enter this position of saponifiable glycerolipids *via* GP, as well. Thus, the ^{3}H/^{14}C ratio at C-2 of ether-containing glycerolipids should be maximal, since, in this case, ^{14}C entry is confined to acyl DHAP. The ratio in saponifiable glycerolipids could range from zero to that found in the ether-containing glycerolipids, depending on the fraction of lipid glycerol synthesized *via* acyl DHAP.

The presence of ^{3}H at C-2 of GP (Table 2) complicates the analysis since some ^{3}H can apparently enter C-2 of glycerolipids by the GP pathway. The magnitude of this complicating factor depends on whether the ^{3}H/^{14}C ratio at C-2 of GP observed at the end of the 30 min incubation accurately reflects the value of this ratio throughout the incubation. Further studies are in progress to measure the ^{3}H/^{14}C ratios at C-2 of the four glycerol moieties after shorter incubation periods, but preliminary results indicate that this ratio in GP is also small after 15 min of incubation.

Assuming that glycerol in ether-containing glycerolipids enters only *via* acyl DHAP and that synthesis of both ether-containing and saponifiable glycerolipids occurs in the same cellular compartment, it can be seen from the data in Table 2 that a significant fraction of the glycerol in saponifiable glycerolipids enters *via* acyl DHAP. For the incubation with [3-^{3}H, U-^{14}C]glucose, the ^{3}H/^{14}C ratios at C-2 of glycerol in GP, saponifiable lipids, and plasmalogens are consistent with 55% of the saponifiable glycerolipids having been synthesized *via* acyl DHAP. In contrast, Katz and Rognstad report that [3-^{3}H]glucose

did not significantly label C-2 of the glycerol in neutral glycerolipids from epididymal fat pads of rats (6). The reason for this difference is unclear but may relate to a tissue difference or a characteristic of triglycerides. Using various methods, the presence (1,22) and absence (23) of the acyl DHAP pathway in liver have been claimed. The further use of variously labeled glucose precursors should permit quantitative evaluation of the alternative routes from glucose to lipid glycerol.

Acknowledgments. Supported by Grants NDS 03101, MH 07417, and NS 08841 from the National Institutes of Health and U.S.P.H.S. Grant CA 13978-02. We thank Barbara Fishel for helping with some of the cell cultures.

REFERENCES

1. Agranoff, B. W. and Hajra, A. K. (1971) Proc. Nat. Acad. Sci., U.S., 68, 411-415.
2. Hajra, A. K. (1973) Tumor Lipids: Biochemistry and Metabolism, pp. 183-199, Amer. Oil Chem. Soc. Press, Champaign.
3. Van den Bosch, H. (1974) Ann. Rev. Biochem., 43, 243-277.
4. Borrebaek, B., Abraham, S., and Chaikoff, I. L. (1965) Biochim. Biophys. Acta, 96, 237-247.
5. LaBelle, E. F. and Hajra, A. K. (1972) J. Biol. Chem., 247, 5825-5834.
6. Katz, J. and Rognstad, R. (1966) J. Biol. Chem., 241, 3600-3610.
7. Katz, J. and Wals, P.A. (1972) Biochem. J., 128, 879-899.
8. Katz, J., Rognstad, R., and Kemp, R. G. (1965) J. Biol. Chem., 240, PC1484-PC1486.
9. Kemp, R. G. and Rose, I. A. (1964) J. Biol. Chem., 239, 2998-3005.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.
11. Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol., 37, 911-917.
12. LePage, G. A. (1957) Manometric Techniques, pp. 268-281, Burgess Publishing Co., Minneapolis.
13. Seiffert, U. B. and Agranoff, B. W. (1965) Biochim. Biophys. Acta, 98, 574-581.
14. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem., 226, 497-509.
15. Snyder, F., Blank, M. L., and Wykle, R. L. (1971) J. Biol. Chem., 246, 3639-3645.
16. Wood, R. and Snyder, F. (1968) Lipids, 3, 129-135.
17. Dawson, R. M. C. (1967) Lipid Chromatographic Analysis, vol. I, pp. 163-189, Marcel Dekker, Inc., New York.
18. Hanahan, D. J. (1965) J. Lipid Res., 6, 350-355.
19. Renkonen, O., Gahmberg, C. G., Simons, K., and Kaariainen, L. (1972) Biochim. Biophys. Acta, 255, 66-78.
20. Laine, R., Kettunen, M., Gahmberg, C. G., Kaariainen, L., and Renkonen, O. (1972) J. Virol., 10, 433-438.
21. Friedberg, S. J. and Heifetz, A. (1975) Biochemistry, 14, 570-574.
22. Manning, R. and Brindley, D. N. (1972) Biochem. J., 130, 1003-1012.
23. Rognstad, R., Clark, D. G., and Katz, J. (1974) Biochem. J., 140, 249-251.