Pathways of Glyceride Glycerol Synthesis

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Isolated rat liver parenchymal cells were incubated for various periods with [U- ^{14}C ,2- ^3H]glycerol and the radioisotopic yields in the major products were determined, as well as the $^3\text{H}/^{14}\text{C}$ ratios in glyceride glycerol and intracellular glycerol phosphate. Under the conditions used (0.1 mM-glycerol+10 mM-L-lactate or 10 mM-glycerol as substrates), only small differences were found between these $^3\text{H}/^{14}\text{C}$ ratios. The results suggest a minor role for a pathway of glyceride glycerol synthesis involving reduction of acylated dihydroxyacetone phosphate, under these experimental conditions.

The major pathway for the biosynthesis of the glycerol moiety of lipids has long been considered to be via *sn*-glycerol 3-phosphate. However, evidence has been produced for another pathway involving acylation of dihydroxyacetone phosphate and subsequent reduction with NADPH (Hajra & Agranoff, 1967). Manning & Brindley (1972) have attempted to measure the two pathways in rat liver slices. They compared the $^3\text{H}/^{14}\text{C}$ ratio in lipid glycerol and intracellular glycerol phosphate, by using [1,3- ^{14}C ,2- ^3H]glycerol as substrate. From the markedly higher ratio in glycerol phosphate, they calculated that the dihydroxyacetone phosphate pathway was a major pathway of lipid glycerol synthesis. Though we have obtained data which are similar in some respects, our interpretation of our experiments suggests little or no participation of a dihydroxyacetone phosphate pathway under the conditions we have used.

Methods

Male rats (200 g) were meal-fed once a day (6.00 to 9.00 a.m.) and killed 4 h after the last meal. Isolated rat liver cells were prepared essentially by the procedure of Berry & Friend (1969). The washed cells were diluted about fourfold in Krebs & Henseleit (1932) buffer, and 2 ml of this, containing about 60 mg (Expt. 1) or 80 mg (Expt. 2) defatted dry wt. of cells, was incubated at 38°C in 50 ml Erlenmeyer flasks with the radioactive and non-radioactive substrates and with O_2+CO_2 (95:5) as the gas phase. About 3 μCi of [U- ^{14}C]glycerol and 50 μCi of [2- ^3H]glycerol were used. In Expt. 1 (Table 1) 0.1 mM-glycerol was used in the presence of 10 mM-L-lactate; in Expt. 2, 10 mM-glycerol was used in the absence of lactate. The vessels were capped with rubber serum stoppers from which plastic wells for CO_2 collection were suspended. Incubations were terminated by adding 0.5 ml of 0.5 M- H_2SO_4 to

the medium and 0.25 ml of phenethylamine-water (1:1, v/v) to the hanging well. The cells and medium were made to 10.0 ml with water and centrifuged. At this stage, [U- ^{14}C]glycerol phosphate was added to the medium from one incubation which contained no radioactive substrate, to aid in the isolation of intermediate glycerol phosphate. A portion (8 ml) was applied to a column (1 cm $\times$ 5 cm) of Amberlite CG-120 (H^+ form; 100-200 mesh) which was on top of a column (1 cm $\times$ 11 cm) of Dowex 1 (X8; acetate form; 200-400 mesh). The columns were washed with water until 30 ml of eluate was collected. Duplicate 1.0 ml samples of this neutral fraction were used to determine radioactivity, one of them being first dried in a vacuum desiccator. The difference in radioactivity between these samples was taken as the yield of ^3HHO . A 25 ml portion of the 30 ml neutral fraction was evaporated to dryness and applied to a column (1 cm $\times$ 6 cm) of Dowex 1 (X8; borate form; 100-200 mesh), which was then washed with water. Residual glycerol was eluted with 15 mM- $\text{Na}_2\text{B}_4\text{O}_7$ and then glucose was eluted with 2 M-acetic acid. Controls with [^{14}C]glycerol and [^{14}C]glucose were used to follow the separation.

Lactate was eluted from the 1 cm $\times$ 11 cm column of Dowex 1 (X8; acetate form) with 2 M-acetic acid and then a crude fraction containing glycerol phosphate was eluted with 4 M-formic acid. This latter fraction was evaporated to dryness in a stream of air and incubated for 24 h with 5 mg (10 units) of acid phosphatase (EC 3.1.3.2) in 2 ml of 0.25 M-sodium acetate buffer, pH 5.0. The resulting solution was put through a column (1 cm $\times$ 5 cm) of Dowex 1 (X8; acetate form; 200-400 mesh), which would retain any organic acids, and the water-wash was again evaporated to dryness. The glycerol obtained from the dephosphorylation of glycerol phosphate was then further purified by trapping on a borate column as used in the previous paragraph for the isolation of residual glycerol.

Lipids were extracted and saponified as described elsewhere (Katz *et al.*, 1966). The acidified aqueous layer containing the glyceride glycerol was applied to a column (1cm×3cm) of Amberlite CG-120 (H⁺ form; 100–200 mesh) which was on top of a column of Dowex 1 (X8; acetate form; 100–200 mesh) and the columns were washed with water until 20ml of eluate was collected. This fraction, containing about 80% of the radioactivity applied to the column, was evaporated to dryness, made to a small volume, and a sample (1 ml) was used to determine the radioactivity. The remainder of the radioactivity probably is in glycerol phosphate or glycerol phosphoryl derivatives produced from the alkaline hydrolysis of phospholipids.

The radioactivity in the fractions was determined by liquid scintillation counting. Scintillation fluid (10ml), consisting of toluene–Triton X-100 (3:1, v/v) containing 5g of 2,5-diphenyloxazole and 60mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l, was added to 1ml portions of aqueous samples.

[U-¹⁴C]Glycerol was synthesized by enzymic dismutation of [U-¹⁴C]glucose to 3-phosphoglycerate and glycerol phosphate (Schmidt *et al.*, 1970), followed by dephosphorylation and purification by chromatography on Whatman 3MM paper with butan-1-ol–acetic acid–water (4:1:2, by vol.). [2-³H]-Glycerol was made by reduction of dihydroxyacetone with NaB³H₄ at pH7. *sn*-[U-¹⁴C]Glycerol 3-phosphate was obtained by phosphorylation of [U-¹⁴C]-glycerol with ATP catalysed by glycerol kinase (EC 2.7.1.30) (Genovese *et al.*, 1970). Enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Results and Discussion

The approach of Manning & Brindley (1972) to measure quantitatively the pathways of lipid glycerol synthesis is based on the use of [¹⁴C]glycerol and [2-³H]glycerol. The ³H will be retained in glycerol phosphate and during its subsequent conversion into lipid glycerol. However, the ³H is transferred to NAD or flavin during the formation of dihydroxyacetone phosphate. Subsequent incorporation of dihydroxyacetone phosphate into lipid glycerol involves reduction of the acylated dihydroxyacetone phosphate with NADPH and very little reincorporation of ³H would be expected. Thus ³H from [2-³H]glycerol will be incorporated into lipid glycerol synthesized only by the glycerol phosphate pathway, whereas ¹⁴C from glycerol will be incorporated into lipid glycerol by either pathway.

In Expt. 1 of Table 1 we used [U-¹⁴C,2-³H]glycerol at a concentration of 0.1mm, which was the concentration used by Manning & Brindley (1972). The major differences were that we used isolated rat liver cells instead of slices, and used 10mm-lactate as the

Table 1. Radioisotope yields and ³H/¹⁴C ratios during incubation of liver cells with [U-¹⁴C,2-³H]glycerol

Expt. no.	Initial concn. of glycerol (mm)	Incubation time (min)	Radioisotopic yields (% of added radioactivity)												³ H/ ¹⁴ C ratios			
			³ H	¹⁴ CO ₂	Glucose		Lactate		Residual glycerol		Glyceride glycerol		Glycerol phosphate					
					³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C		
1	0.1	20	48.2	3.2	8.9	55.0	43.4	16.1	1.5	1.7	3.1	3.1	0.63	0.39	1.00	1.64		
		40	67.8	6.3	11.5	59.1	22.4	10.5	1.3	1.4	3.3	3.3	0.36	0.15	1.03	2.40		
		60	80.6	8.5	12.1	62.0	9.2	6.2	1.2	1.4	3.0	2.9	0.31	0.069	1.04	4.50		
2	10	5	4.7	0.68	2.7	1.9	2.9	2.0	81.6	84.7	0.17	0.16	4.0	3.8	1.03	1.06		
		10	6.7	0.10	3.1	8.7	5.2	2.5	71.4	75.9	0.44	0.42	6.8	6.1	1.05	1.12		
		20	13.6	0.38	5.3	28.9	8.4	2.8	50.0	54.7	1.17	1.10	9.9	7.3	1.07	1.36		
		40	44.1	0.80	12.8	74.7	12.1	2.6	5.3	3.8	2.9	2.4	10.0	4.5	1.28	2.23		
		60	66.6	1.46	14.5	79.8	2.2	1.5	1.1	0.5	3.6	2.6	1.2	0.23	1.39	5.30		

Isolated rat liver parenchymal cells were incubated in bicarbonate-buffered medium (2ml) for the times indicated. In Expt. 1 only, L-lactate (10mm) was the major substrate. The products were isolated as described in the Methods section. The ³H/¹⁴C ratios are the ratios of the percentage yields; this is equivalent to taking the ³H/¹⁴C ratio in the substrate as equal to 1.0. Incorporation of ¹⁴C and ³H into fatty acids of lipids was about 10–15% of that in glyceride glycerol (at 60min) in both experiments.

major substrate. We found, as did they, that the $^3\text{H}/^{14}\text{C}$ ratio in glycerol phosphate increased during incubation. However, we determined the major products of metabolism of radioactive material in this experiment and found that even at the earliest time investigated the radioactive substrate was essentially depleted, with about half of the ^3H from the $[2\text{-}^3\text{H}]\text{glycerol}$ appearing in water together with a large amount in lactate and lesser amounts in glucose and glyceride glycerol. Subsequent ^3H metabolism apparently involves mainly the conversion of ^3H in lactate into glucose and water. The major synthesis of radioactive lipid glycerol occurred within the first 20 min. The $^3\text{H}/^{14}\text{C}$ ratio in glycerol phosphate, even at the shortest (20 min) incubation period, thus has no direct relation to the $^3\text{H}/^{14}\text{C}$ ratio in the initial substrate, and in fact in this experiment probably reflects in large part the relative rates of exchange of ^3H and ^{14}C between lactate and glycerol phosphate. It is evident that the experimental conditions we employed are not suitable to measure the relative pathways of lipid glycerol synthesis by a comparison of $^3\text{H}/^{14}\text{C}$ ratios.

To find more suitable conditions for measuring the pathways by using the $^3\text{H}/^{14}\text{C}$ ratio method, we used $[\text{U-}^{14}\text{C}, 2\text{-}^3\text{H}]\text{glycerol}$ at a higher concentration (10 mM) to obtain data before the depletion of the radioactive substrate (Expt. 2, Table 1). About half of the radioactive substrate remained after 20 min, but it was nearly depleted in the 20–40 min period. A smaller proportion of the ^3H was converted into lactate in this experiment, in which no lactate was added initially (although some lactate accumulation occurred, as shown by analysis). Comparison of the $^3\text{H}/^{14}\text{C}$ ratios in lipid glycerol and glycerol phosphate shows some slight differences during the first 20 min, but this is of questionable significance. It should be remembered that the glycerol phosphate ratio reflects one point in time, whereas the glyceride glycerol ratio is a cumulative value reflecting synthesis occurring up to that point in time. Again the major divergence between the $^3\text{H}/^{14}\text{C}$ ratios in glycerol phosphate and glyceride glycerol occurs only after the radioactive substrate becomes largely depleted. The data suggest at most a quite minor pathway of glyceride glycerol synthesis involving dihydroxyacetone phosphate, at least under the conditions of our experiment.

Another approach to measure the dihydroxyacetone phosphate pathway would be to use substrates that generate NADP^3H and to determine the extent of ^3H incorporation on C-2 of glyceride

glycerol. Thus in adipose tissue $[3\text{-}^3\text{H}]\text{glucose}$, under conditions of high lipogenesis, causes extensive radioactive labelling of fatty acids as a result of the generation of NADP^3H in the 6-phosphogluconate dehydrogenase (EC 1.1.1.44) reaction (Katz & Rognstad, 1966). A small amount of radioactivity from $[3\text{-}^3\text{H}]\text{glucose}$ also appears in glyceride glycerol. However, in adipose tissue at least, this does not appear to involve a dihydroxyacetone phosphate pathway of the type discussed above. Rather the radioactive labelling occurs by a pathway in which the ^3H remains bound to carbon, involving conversion of $[3\text{-}^3\text{H}]\text{fructose diphosphate}$ into $[1\text{-}^3\text{H}]\text{dihydroxyacetone phosphate}$ and $[1\text{-}^3\text{H}]\text{glycerol phosphate}$. Evidence for this is that periodate degradation of the glyceride glycerol formed from $[3\text{-}^3\text{H}]\text{glucose}$ shows essentially all of the radioactivity in the formaldehyde derivative (Table 3 of Katz & Rognstad, 1969).

Our results clearly differ from those of Manning & Brindley (1972); in their experiments, which used liver slices rather than cells, 0.1 M-glycerol was added as the only substrate, but, perhaps because less tissue was used, it was not exhausted during the incubation. It is possible that the use of 10 mM-lactate or 10 mM-glycerol in our experiments may have increased the cytoplasmic NADH/NAD^+ ratio and so favoured the glycerol phosphate pathway; the relative importance of this and of the dihydroxyacetone phosphate routes under conditions *in vivo* remains to be clarified.

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