Tritium Isotope Effects in the Measurement of the Glycerol Phosphate and Dihydroxyacetone Phosphate Pathways of Glycerolipid Biosynthesis in Rat Liver

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1. Rat liver slices were employed to study the relative rates of incorporation of a mixture of [2-³H]- or [1,3-³H]-glycerol and [1-¹⁴C]glycerol into lipids. 2. With 0.1 mM-glycerol approx. 82% of the newly synthesized lipid, calculated from ¹⁴C incorporation, was present as neutral lipid, 13% as phosphatidylcholine and 5% as phosphatidylethanolamine. Increasing the glycerol concentration to 40 mM caused a decrease in the percentage of neutral lipid to 59% and a corresponding increase in the percentage of phosphatidylcholine to 36% of the newly synthesized lipid. 3. The (d.p.m. of 2-³H)/(d.p.m. of 1-¹⁴C) ratio in glycerolipid was considerably higher than that in precursor glycerol throughout the range of experimental conditions. In contrast the incorporation of a mixture of [1,3-³H]glycerol and [1-¹⁴C]glycerol into lipid occurred with little or no change in the ³H/¹⁴C ratio. 4. Respiring rat liver mitochondria were found to oxidize a mixture of *sn*-[2-³H]- and *sn*-[1-¹⁴C]-glycerol 3-phosphate with a resultant increase in the ³H/¹⁴C ratio of the remaining *sn*-glycerol 3-phosphate. This increase is due to a ³H isotope effect of the mitochondrial *sn*-glycerol 3-phosphate dehydrogenase (EC 1.1.99.5), which discriminates against *sn*-[2-³H]glycerol 3-phosphate during oxidation. 5. A method is described for the simultaneous determination of the relative contributions of the glycerol phosphate and dihydroxyacetone phosphate pathways of glycerolipid biosynthesis in rat liver slices. The method involves measurement of the (d.p.m. of 2-³H)/(d.p.m. of 1-¹⁴C) ratio in both *sn*-glycerol 3-phosphate and glycerolipid after incubation of rat liver slices with a mixture of [2-³H]glycerol and [1-¹⁴C]glycerol for various times. 6. By using this method it was shown that 40–50% of the glycerol incorporated into lipid by rat liver slices proceeded via the *sn*-glycerol 3-phosphate pathway and 50–60% was incorporated via dihydroxyacetone phosphate.

sn-Glycerol 3-phosphate has been implicated in the biosynthesis of glycerolipids by subcellular fractions from mammalian liver (Kornberg & Pricer, 1953; Kennedy, 1957), and this pathway has been the subject of numerous investigations. Recently, however, it has been shown that dihydroxyacetone phosphate may also be incorporated into glycerolipids, via acyldihydroxyacetone phosphate (Hajra & Agranoff, 1967, 1968a,b; Hajra, 1968). Glyceraldehyde 3-phosphate is also incorporated into glycerolipid, but must first be converted into dihydroxyacetone phosphate (Puleo *et al.*, 1970).

Several investigators have attempted to determine the relative importance of the glycerol phosphate and the dihydroxyacetone phosphate pathways of glycerolipid biosynthesis in bacteria or rat liver slices by measuring the relative incorporations of [1-¹⁴C]-glycerol and [2-³H]glycerol into lipids (Plackett &

Rodwell, 1970; Hill & Lands, 1970; Okuyama & Lands, 1970). These workers reasoned that the 2-³H-labelled atom would be labilized if *sn*-glycerol 3-phosphate was converted into dihydroxyacetone phosphate before esterification, and that any decrease in the (d.p.m. of 2-³H)/(d.p.m. of 1-¹⁴C) ratio (2-³H/1-¹⁴C ratio) in the newly synthesized lipid (compared with precursor glycerol) would be a direct measurement of the relative activities of the two pathways. Surprisingly, all of these investigators recorded an increase in the 2-³H/1-¹⁴C ratio in lipid. Plackett & Rodwell (1970) suggested that the increased ratio observed with *Mycoplasma* strain Y was due to a tritium effect of the *sn*-glycerol 3-phosphate oxidase (EC 1.1.99.5). Other workers failed to explain the increased ratio in lipid and concluded that the dihydroxyacetone phosphate pathway is unimportant in glycerolipid biosynthesis (Hill & Lands, 1970; Okuyama & Lands, 1970).

The present investigation was undertaken in an attempt to explain the increased 2-³H/1-¹⁴C ratio in

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lipid, and to assess whether a measurement of the relative incorporations of [2-³H]glycerol and [1-¹⁴C]-glycerol into lipid could be used to differentiate between the glycerol phosphate and dihydroxyacetone phosphate pathways.

Materials and Methods

Animals and chemicals

Male Wistar rats (200–300g) were obtained from Nottingham University Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K., and were allowed free access to food and water. ATP was purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. The following compounds were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.: oligomycin, dinitrophenol, rotenone, glucose 6-phosphate (sodium salt), glucose 1-phosphate (sodium salt), fructose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), ribose 5-phosphate (sodium salt), *rac*-glycerol 3-phosphate (sodium salt), D-glyceraldehyde, dihydroxyacetone, Dowex resins and the dimethylketal dicyclohexylamine salt of dihydroxyacetone phosphate. Free dihydroxyacetone phosphate was generated from the last-named according to instructions from the suppliers. Silicic acid (100–200 mesh) was purchased from Mallinckrodt Chemical Works, New York, N.Y., U.S.A. and silica gel G from Macherey, Nagel and Co., Düren, West Germany.

Specifically labelled glycerols were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and were used to prepare *sn*-glycerol 3-phosphate by the method of Smith & Hübscher (1966).

Lipid synthesis by rat liver slices

Rats were killed by a blow on the neck and the livers were perfused *in situ* via the aorta (Vavrečka *et al.*, 1969) with cold oxygenated Krebs–Ringer phosphate buffer, pH 7.4 (Dawson, 1969). Livers were removed and slices (0.4 mm thick) were prepared with a McIlwain tissue chopper. Liver slices were blotted, weighed and transferred to tubes containing oxygenated Krebs–Ringer phosphate buffer, pH 7.4, at a tissue concentration of 100 ± 10 mg (wet wt.)/ml of incubation medium. After a 10 min preincubation period at 37°C a mixture of [³H]glycerol and [1-¹⁴C]-glycerol was added and the tubes were incubated at 37°C with shaking in an atmosphere of O₂. In some experiments, where only the radioactive lipid was examined, the reaction was stopped by addition of 50 µl of 11 M-HCl, the slices were disrupted at 4°C by sonication for 8 min at 21 kHz and lipid was extracted by the method of Hajra *et al.* (1968). In other experiments, where water-soluble products were also examined, the reaction was stopped by rapid filtration

under suction; the tissue was immediately washed at 0°C with 15 ml of 0.9% NaCl and rapidly placed in 1.8 ml of methanol–water (5:4, v/v) at 0°C. The slices were sonicated, and lipid was extracted by the method of Bligh & Dyer (1959), which gave the same recoveries of radioactivity as the method of Hajra *et al.* (1968).

Samples of lipid extracts were evaporated to dryness at 60°C in a shaking water bath, dissolved in 10 ml of xylene containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre, and radioactivity was measured by liquid-scintillation counting. In addition lipids were separated into various classes by t.l.c. on silica gel G by using chloroform–methanol–water (65:25:4, by vol.) for resolution of phospholipid classes, and light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (60:40:1, by vol.) for resolution of neutral lipid classes. Spots were detected with iodine vapour, decolourized by exposure to air, scraped from the plates and eluted with chloroform–methanol (1:4, v/v) at 40°C. After evaporation of solvent, radioactivity was measured by liquid-scintillation counting as described above.

Isolation of sn-glycerol 3-phosphate

The top phase from the Bligh & Dyer (1959) extraction was used to isolate the water-soluble phosphates derived from the tissue slices. To the top phase was added 5 µmol of *rac*-glycerol 3-phosphate, 100 µmol of CaCl₂ and 18 ml of acetone–ethanol (5:4, v/v). The mixture was left at –20°C overnight and then centrifuged at –15°C for 30 min at 26000g (*r*_{max} 10.8 cm) and the supernatant was discarded. The precipitate was carefully dried under N₂, mixed with 2 ml of water and 1 g of Dowex 50 (X8; 200–400 mesh; H⁺ form) and left for 20 min at room temperature. The resin was removed by centrifugation, washed twice with 3 ml of water and the supernatants were combined. In order selectively to hydrolyse dihydroxyacetone phosphate and other alkali-labile phosphate esters sufficient 6 M-NaOH was added to give a final concentration of 0.2 M; the tubes were incubated at 60°C for 1 h, cooled and then neutralized by the addition of Dowex 50 (X8; H⁺ form). The resin was again removed by centrifugation and washed with 2 ml of water. To the combined supernatants was added 1 g of Dowex 1 (X8; 200–400 mesh; formate form) and the mixture was left for 20 min at room temperature. The resin was removed by centrifugation, washed twice with 3 ml of water and all supernatants were discarded. Alkali-stable phosphates, such as glycerol phosphate, were eluted from the anion-exchange resin by three successive washes with 3 ml of 2 M-formic acid at 40°C and the supernatants were combined. Aq. formic acid was removed by rotary evaporation under reduced

pressure followed by freeze-drying. The residue was dissolved in 0.5 ml of water and a 150 μ l sample was subjected to descending paper chromatography on Whatman 3 MM paper by two successive developments in di-isopropyl ether–butan-1-ol–90% (w/v) formic acid (3:3:2, by vol.; Wood, 1961). Alternatively, papers were subjected to a two-dimensional development. Separation in the first dimension involved electrophoresis at 3000 V for 45 min in acetic acid–99% (w/v) formic acid–water (87:25:888, by vol.), pH 1.9. After drying overnight the paper was rotated through 90° and subjected to descending chromatography in butan-1-ol–propionic acid–water (98:47:61, by vol.) for 16 h. Since equivalent results were obtained with the one-dimensional and two-dimensional methods of separation the former method was always employed.

Phosphate spots were detected by an acid-molybdate spray (Sheltawy & Dawson, 1969) and then decolourized with NH_3 vapour (Shultz & Johnston, 1971). Spots were eluted with 3 ml of water for 1 h with constant shaking, and the radioactivity in 1.5 ml samples of the eluate was measured by liquid-scintillation counting in 15 ml of Triton X-100–xylene (1:2, v/v) containing 5.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre (Turner, 1969).

Experiments with isolated mitochondria

Respiring mitochondria were isolated from a 10% (w/v) homogenate of rat liver in 0.25 M-sucrose containing 1 mM-EGTA [ethanedioxybis(ethyl-amine)tetra-acetic acid] and 4 mM-tris, adjusted to pH 7.4 with HCl. The homogenate was centrifuged for 10 min at 667 g (r_{max} , 10.8 cm) to remove nuclei and cell debris. The supernatant was centrifuged for 10 min at 5000 g (r_{max} , 10.8 cm) and the mitochondrial pellet was washed twice by gentle resuspension in the homogenizing medium and further centrifugation for 10 min at 5000 g . All operations were performed at 4°C. Protein was determined by a biuret method (Brindley & Hübscher, 1965).

At the times indicated in Fig. 4, 10 μ l samples of medium were added to 50 μ l of 1 M-NaOH and incubated at 40°C for 60 min to hydrolyse dihydroxyacetone phosphate; under these conditions dihydroxyacetone is converted into a mixture of hexoses (Guthrie & Honeyman, 1964). The solution was then neutralized with 1 M-HCl and 50 μ l samples were taken for chromatography. The radioactive compounds were separated by descending chromatography on Whatman 3 MM paper with phenol–water–acetic acid (74:26:1, w/v/v), containing 1 mM-sodium EDTA (Moses, 1969), or methanol–90% (w/v) formic acid–water (40:7:3, by vol.) as the developing solvents. Phosphates were detected and radioactivity was measured as described above. Standard ketones

and aldehydes were detected with 2,4-dinitrophenylhydrazine (Davenport, 1969) and alkali-treated authentic dihydroxyacetone was detected by an aniline–diphenylamine spray (Bailey, 1969). Corresponding spots were cut from test lanes, eluted with 3 ml of water and assayed for radioactivity as described for *sn*-glycerol 3-phosphate.

The use of triose, pentose and hexose phosphates and their free sugars in pilot experiments indicated that *sn*-glycerol 3-phosphate could be separated from these compounds by the procedures described above.

Degradation of labelled lipid

Lipids from 200 mg (wet wt.) of liver slices were applied in chloroform to a 3 g silicic acid column (100–200 mesh); neutral lipid was eluted with 20 ml of chloroform, and phospholipid with 20 ml of chloroform–methanol (1:9, v/v). After removal of solvent, neutral lipid fractions were saponified at 85°C for 2 h in 1 ml of 2 M-NaOH. Samples were cooled, 3 ml of water was added and non-saponifiable lipid was removed by two successive extractions with 3 ml of light petroleum (b.p. 40–60°C). The aqueous phase was adjusted to pH 6 by addition of Dowex 50 (X8; H^+ form) and then to pH 2 with 1 M- H_2SO_4 . Fatty acids were removed by three successive extractions with 3 ml of light petroleum (b.p. 40–60°C).

A sample of the aqueous phase was mixed with 400 μ g of glycerol and subjected to ascending paper chromatography on Whatman no. 2 paper in ethanol – aq. NH_3 (sp.gr. 0.88) – water (20:1:4, by vol.). Lanes containing non-radioactive glycerol as a marker were stained with a Phenol Red–borate buffer spray (Bailey, 1969). Corresponding spots were cut from test lanes and eluted with 3 ml of water, and radioactivity was determined as described above.

After extraction of fatty acids from the aqueous phase, the Dowex resin was washed twice with 2 ml of water, and the supernatants were combined and mixed with 100 μ mol of glycerol and 0.9 ml of freshly prepared 10% (w/v) NaIO_4 . The tubes were tightly stoppered and left at room temperature for 1 h, after which each sample was cooled to 4°C and passed down a column (1 cm $\times$ 8 cm) of Dowex 1 (X8; HCO_3^- form). The column was washed with 25 ml of water and the combined aqueous eluate was mixed with 35 ml of 0.4% (w/v) dimedone and stored overnight at 4°C.

Under these conditions formaldehyde, derived from C-1 and C-3 of glyceride glycerol, is trapped as insoluble methylene dimedone (Saunders & Dawson, 1962). The precipitate was isolated by filtration under reduced pressure, washed well with ice-cold water, and allowed to dry. The methylene dimedone was dissolved in 60 ml of xylene containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre and assayed for radioactivity by

liquid-scintillation counting. Since methylene dimedone is self-quenching it was diluted with sufficient scintillant to obtain high counting efficiencies.

All radioactivity measurements were performed in a Packard liquid-scintillation spectrometer model 3375; counting efficiency was monitored by use of an external standard ratio (Turner, 1969), and the results were processed by a PDP/8L Digital Computer.

Results

Conditions for the incorporation of glycerol into lipids by rat liver slices

Preliminary experiments were performed to establish optimum conditions for the incorporation of [1,3-³H]glycerol into lipids by rat liver slices. Variation of the glycerol concentration indicated that optimum incorporation into lipid occurred at a concentration of 10 mM and greater, confirming the results of Liberti & Jezyk (1970). The rate of glycerol incorporation, at a given concentration, remained constant for at least 90 min, and was approx. 3.5 nmol/h per 100 mg of liver at 0.1 mM-glycerol and 30 nmol/h per 100 mg of liver at 10 mM-glycerol. The addition of 1 mM-palmitate had little effect on the incorporation of glycerol into glycerolipid by rat liver slices.

The effect of different buffers (Dawson, 1969; Liberti & Jezyk, 1970), all at pH 7.4, was also examined. In contrast to the results of Liberti & Jezyk (1970), Krebs-Ringer tris buffer (equilibrated with O₂) was found to give approx. 30% lower incorporation of glycerol into lipid, compared with either Krebs-Ringer phosphate buffer (equilibrated with O₂) or Krebs-Ringer bicarbonate buffer (equilibrated with O₂ + CO₂ (95:5)). Oxygenated Krebs-Ringer phosphate buffer, pH 7.4, was employed in all subsequent experiments.

Incorporation of mixtures of [2-³H]- or [1,3-³H]-glycerol and [1-¹⁴C]glycerol into lipids by rat liver slices

The relative rates of incorporation of [2-³H]- or [1,3-³H]-glycerol and [1-¹⁴C]glycerol into lipids by rat liver slices were compared. The results are expressed in terms of the (d.p.m. of ³H)/(d.p.m. of ¹⁴C) ratio (³H/¹⁴C ratio) as a function of either time (Fig. 1) or exogenous glycerol concentration (Fig. 2).

The magnitude of the ³H/¹⁴C ratio of glycerolipid classes synthesized from a mixture of [2-³H]glycerol and [1-¹⁴C]glycerol by rat liver slices depended on the time of incubation (Fig. 1) and the concentration of exogenous glycerol (Fig. 2). This ratio was greater than that in the precursor glycerol mixture throughout the range of experimental conditions. Glycerolipid synthesized from 10 mM-glycerol was shown to have a lower 2-³H/1-¹⁴C ratio than that synthesized

from 0.1 mM-glycerol at corresponding times during a 60 min incubation period (Fig. 1). In addition, the 2-³H/1-¹⁴C ratios of neutral lipid and phosphatidylcholine, at a given glycerol concentration, were only slightly different in all of the cases investigated (Figs. 1 and 2). The amount of radioactivity incorporated into phosphatidylethanolamine, which accounted for only 5% of the newly synthesized lipid, was low and accurate ³H/¹⁴C ratios could not be calculated. However, in combination with the analysis of 2-³H/1-¹⁴C ratios in phosphatidate (Okuyama & Lands, 1970), the results shown in Figs. 1 and 2 suggest that all glycerolipid classes newly synthesized by rat liver slices from a mixture of [2-³H]glycerol and [1-¹⁴C]glycerol exhibit a similar elevated ³H/¹⁴C ratio.

In contrast, the incorporation of a mixture of [1,3-³H]glycerol and [1-¹⁴C]glycerol into lipid occurred with little or no change in the ³H/¹⁴C ratio, which was independent of incubation time (Fig. 1) and exogenous glycerol concentration (Fig. 2). The small decrease of about 5%, which sometimes occurred in the 1,3-³H/1-¹⁴C ratio (Fig. 1), probably reflects a

Fig. 1. Effect of incubation time on the relative incorporation of [1,3-³H]-, [2-³H]-glycerol and [1-¹⁴C]-glycerol into lipids

Slices of rat liver (100 mg wet wt.) were incubated as described in the Materials and Methods section for the times indicated. Glycerol addition was as follows: system A, 0.1 mM-glycerol containing 4 μ Ci of [1,3-³H]glycerol and 2 μ Ci of [1-¹⁴C]glycerol; system B, 0.1 mM-glycerol containing 4 μ Ci of [2-³H]glycerol and 2 μ Ci of [1-¹⁴C]glycerol; system C, 10 mM-glycerol containing 4 μ Ci of [2-³H]glycerol and 2 μ Ci of [1-¹⁴C]glycerol. The ratio (d.p.m. of ³H)/(d.p.m. of ¹⁴C) in lipid fractions is recorded as follows: system A, phosphatidylcholine (Δ) and neutral lipid (Δ); system B, phosphatidylcholine (●) and neutral lipid (○); system C, phosphatidylcholine (■) and neutral lipid (□).

Fig. 2. Effect of glycerol concentration on the relative incorporation of [1,3- ^3H]-, [2- ^3H]- and [1- ^{14}C]-glycerol into lipids

Slices of rat liver (100mg wet wt.) were incubated for 60min as described in the Materials and Methods section at various concentrations of glycerol labelled with (A) 4 μCi of [1,3- ^3H]glycerol and 2 μCi of [1- ^{14}C]glycerol or (B) 4 μCi of [2- ^3H]glycerol and 2 μCi of [1- ^{14}C]glycerol. The ratio (d.p.m. of ^3H)/(d.p.m. of ^{14}C) is recorded as follows: system A, phosphatidylcholine (▲) and neutral lipid (△); system B, phosphatidylcholine (●) and neutral lipid (○).

small isotope effect of one or more of the enzymes involved in the conversion of [1,3- ^3H]glycerol into lipid, causing a slightly slower utilization of the tritiated species.

At a given glycerol concentration, the percentage composition of newly synthesized lipid, calculated from ^{14}C incorporation, remained relatively constant throughout the 60min incubation period. With 0.1mm-glycerol, approx. 82% of ^{14}C -labelled lipid was present as neutral lipid, 13% as phosphatidylcholine and 5% as phosphatidylethanolamine. The newly synthesized neutral lipid comprised approx. 85% triglyceride, 10% diglyceride and 5% monoglyceride. With the exception of phosphatidylethanolamine, the percentage composition of ^{14}C -labelled lipid depended on the concentration of exogenous glycerol (Fig. 3). The percentage of newly synthesized neutral lipid decreased to 59% at 40mm-glycerol, whereas that of phosphatidylcholine increased to 39%. These results are in good agreement with those of Liberti & Jezyk (1970).

The relative rates of incorporation of [2- ^3H]glycerol and [1- ^{14}C]glycerol into lipids by rat liver slices were compared to gain some information about the relative quantitative importance of the glycerol phosphate and dihydroxyacetone phosphate pathways of glycerolipid biosynthesis. However, direct interpretation of the results obtained (Figs. 1 and 2) in terms of these two pathways is impossible because of the unexpected increase in the 2- ^3H /1- ^{14}C ratio in

Fig. 3. Effect of glycerol concentration on the composition of the lipid synthesized by rat liver slices

The incubations were those described in Fig. 2. The synthesis of lipid was determined by measuring incorporation of 1- ^{14}C . Lipid classes are indicated as follows: ●, neutral lipid; ■, phosphatidylcholine; ▲, phosphatidylethanolamine.

glycerolipid products. Similar considerations apply to the ratios obtained in newly synthesized lipid reported by other investigators for bacterial systems (Plackett & Rodwell, 1970; Hill & Lands, 1970) and rat liver slices (Okuyama & Lands, 1970).

In an attempt to resolve the unexpected ratio changes two approaches were taken. First, the possibility that molecular rearrangement of ^3H from [2- ^3H]glycerol results in elevated 2- ^3H /1- ^{14}C ratios in glycerolipid products was examined by degradation of the labelled neutral lipid. The results (Table 1) show that only a small degree of tritium rearrangement occurs during the incorporation of [2- ^3H]glycerol into glycerolipid, and that this is insufficient to account for the magnitude of the observed ratio changes (Figs. 1 and 2). Secondly, the possibility that the elevated 2- ^3H /1- ^{14}C ratio in glycerolipid may be due to a ^3H isotope effect operating on the enzyme responsible for oxidation of *sn*-glycerol 3-phosphate, as suggested by Plackett & Rodwell (1970) for *Mycoplasma* strain Y, was investigated by using mitochondrial preparations from rat liver.

Oxidation of sn-glycerol 3-phosphate by rat liver mitochondria

sn-Glycerol 3-phosphate is known to be a substrate for two dehydrogenases in rat liver. The membrane-bound dehydrogenase (EC 1.1.99.5) present in mitochondria favours the formation of dihydroxyacetone phosphate from *sn*-glycerol 3-phosphate, whereas the soluble dehydrogenase (EC 1.1.1.8) is thought to have an equilibrium constant that favours the reverse reaction (Axelrod, 1967). Consequently, we examined

Table 1. *Degradation of glycerol and neutral lipid*

Samples of neutral lipid synthesized by rat liver slices from mixtures of [2-³H]glycerol and [1-¹⁴C]glycerol or [1,3-³H]glycerol and [1-¹⁴C]glycerol (see Figs. 1 and 2) were degraded as described in the Materials and Methods section. The correspondingly labelled glycerol precursors were taken through the same procedure. Recovery of radioactivity in non-saponifiable lipid and fatty acid was less than 0.6% in all cases.

Source	Recovery (%)				Ratio (d.p.m. of ³ H)/ (d.p.m. of ¹⁴ C)	
	Glycerol (after chromatography)		Methylene dimedone		Original glycerol or lipid	Methylene dimedone
	³ H	¹⁴ C	³ H	¹⁴ C		
[1,3- ³ H]- and [1- ¹⁴ C]-Glycerol	97.0	98.9	93.5	95.5	2.00	1.96
Neutral lipid derived from [1,3- ³ H]- and [1- ¹⁴ C]-glycerol	97.2	98.4	90.8	92.3	1.92	1.89
[2- ³ H]- and [1- ¹⁴ C]-Glycerol	98.5	99.0	0.8	95.0	2.00	0.02
Neutral lipid derived from [2- ³ H]- and [1- ¹⁴ C]-glycerol	97.8	98.5	2.1	91.0	4.22	0.10

Fig. 4. *Oxidation of sn-[1-¹⁴C]glycerol 3-phosphate by rat liver mitochondria*

The incubation system consisted of: 25 mM-tris adjusted to pH 7.4 with HCl, 5 mM-potassium phosphate, pH 7.4, 80 mM-KCl, 1 mM-CaCl₂, 3 mM-MgCl₂, 1 mM-ATP, 1.58 mM-*sn*-glycerol 3-phosphate (containing 10 μCi of *sn*-[2-³H]- and 5 μCi of *sn*-[1-¹⁴C]-glycerol 3-phosphate), 3 μg of oligomycin, 0.3 mM-dinitrophenol, 4 μg of rotenone and 4 mg of mitochondrial protein in a final volume of 1.0 ml. The medium was saturated with air and shaken at 37°C. At the times indicated 10 μl samples were processed as described in the Materials and Methods section. Results are expressed as μmol of *sn*-[1-¹⁴C]glycerol 3-phosphate remaining (■) or dihydroxy[1-¹⁴C]-acetone phosphate produced (●).

the oxidation of *sn*-glycerol 3-phosphate by mitochondrial preparations from rat liver, but the soluble enzyme was not investigated.

Rat liver mitochondria were found to oxidize a mixture of *sn*-[2-³H]- and *sn*-[1-¹⁴C]glycerol 3-phosphate to [1-¹⁴C]dihydroxyacetone phosphate (Fig. 4). Dinitrophenol was added to the incubation to uncouple oxidative phosphorylation, rotenone to inhibit NAD⁺-linked oxidations and oligomycin to inhibit the dinitrophenol-stimulated adenosine triphosphatase. These additions ensured a good rate of *sn*-glycerol 3-phosphate oxidation (Fig. 4).

During the period of oxidation the 2-³H/1-¹⁴C ratio of *sn*-glycerol 3-phosphate rose from its initial value of 2 to 27, owing to the relative inability of the mitochondrial dehydrogenase to oxidize *sn*-[2-³H]-glycerol 3-phosphate (Fig. 5). This conclusion is supported by the work of Carnicero *et al.* (1972), who demonstrated that *sn*-[2-³H]glycerol 3-phosphate is oxidized 2.17 times more slowly than the unlabelled substrate by rat liver mitochondria in the absence of inhibitors and uncouplers.

The experiment described in Figs. 4 and 5 was designed to demonstrate the effect of oxidation on the 2-³H/1-¹⁴C ratio in *sn*-glycerol 3-phosphate, rather than to measure the isotope effect. Accurate estimation of the isotope effect is made difficult by the small degree of ³H labilization that occurred during the initial phase of oxidation. During the first 10 min of oxidation less than 5% of the *sn*-[2-³H]glycerol 3-phosphate was oxidized, compared with 45% of the *sn*-[1-¹⁴C]glycerol 3-phosphate. This would give an isotope effect greater than that reported by Carnicero *et al.* (1972). The discrepancy between the two results may be due to different incubation and assay conditions, particularly with respect to substrate concentration and the presence or absence of inhibitors and uncouplers of oxidative phos-

Fig. 5. Effect of mitochondrial oxidation on the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in the remaining *sn*-glycerol 3-phosphate

The incubation system was that described in Fig. 4. The ratio (d.p.m. of $2\text{-}^3\text{H}$)/(d.p.m. of $1\text{-}^{14}\text{C}$) in the remaining *sn*-glycerol 3-phosphate is plotted as a function of time.

phorylation. At later times of oxidation (over 30 min), when 90–95% of the *sn*-glycerol 3-phosphate had been oxidized and the $2\text{-}^3\text{H}$ -labelled species considerably concentrated, approx. 16–23% of the ^3H was labilized.

Measurement of the relative importance of the sn-glycerol 3-phosphate and dihydroxyacetone phosphate pathways of glycerolipid biosynthesis in rat liver slices

The results presented in Fig. 5 suggest that a ^3H isotope effect of the *sn*-glycerol 3-phosphate dehydrogenase causes an increase in the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio of *sn*-glycerol 3-phosphate, which may act as a precursor for lipid biosynthesis. This effect would explain the elevated $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio that was always observed in lipid products (Figs. 1 and 2).

Under the conditions of the present study ^3H is incorporated into glycerolipid from $[2\text{-}^3\text{H}]\text{glycerol}$ exclusively via the *sn*-glycerol 3-phosphate pathway. If this pathway is the only route of glycerolipid synthesis in rat liver slices one would expect the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in lipid to approximate to that in *sn*-glycerol 3-phosphate. Conversely, if lipid were synthesized exclusively from a metabolite labelled solely with ^{14}C (e.g. dihydroxyacetone phosphate) the

Fig. 6. Effect of incubation time on the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in *sn*-glycerol 3-phosphate and in total glycerol lipid from rat liver slices

Slices of rat liver (200mg wet wt.) were incubated as described in the Materials and Methods section with 0.1mM-glycerol containing $10\mu\text{Ci}$ of $[2\text{-}^3\text{H}]\text{glycerol}$ and $5\mu\text{Ci}$ of $[1\text{-}^{14}\text{C}]\text{glycerol}$. Total lipid (●) and *sn*-glycerol 3-phosphate (■) were analysed and the ratio (d.p.m. of ^3H)/(d.p.m. of ^{14}C) is expressed as a function of time.

$2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in lipid would be zero. In an intermediate situation, however, where lipid may be derived from both *sn*-glycerol 3-phosphate (labelled with $2\text{-}^3\text{H}$ and ^{14}C) and dihydroxyacetone phosphate (labelled solely with ^{14}C) one would expect the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio of lipid products to be lower than that in *sn*-glycerol 3-phosphate.

These predictions were tested in the following manner. Rat liver slices were incubated with a mixture of $[2\text{-}^3\text{H}]\text{glycerol}$ and $[1\text{-}^{14}\text{C}]\text{glycerol}$ at a concentration of 0.1mM, which has been shown to be the normal plasma glycerol concentration in the adult rat (Robinson & Newsholme, 1969). At various time-intervals the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratios of *sn*-glycerol 3-phosphate and total glycerolipid were measured (Fig. 6). Glycerolipid was not resolved into various classes, since it had been shown (Figs. 1 and 2) that the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratios of the principal classes are essentially similar. It was found that, during the first 10 min of incubation, there was a rapid rise in the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio of the *sn*-glycerol 3-phosphate owing, presumably, to the ^3H isotope effect in the oxidation of *sn*-glycerol 3-phosphate. This ratio was found to be considerably higher than the cumulative $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in glycerolipid synthesized throughout the corresponding time-period (Fig. 6). This indicates, by the above reasoning, that *sn*-glycerol 3-phosphate is not the only precursor of glycerolipid in rat liver slices and that another metabolite, labelled solely with ^{14}C

in the present study, is also incorporated. Present evidence from work with subcellular fractions (Hajra & Agranoff, 1967, 1968a,b; Hajra, 1968; Puleo *et al.*, 1970) indicates that this metabolite is dihydroxyacetone phosphate. Glyceraldehyde 3-phosphate is also incorporated into glycerolipid, but must first be converted into dihydroxyacetone phosphate (Puleo *et al.*, 1970). If dihydroxyacetone phosphate is the ^{14}C -labelled precursor then the quantitative importance of the *sn*-glycerol 3-phosphate and dihydroxyacetone phosphate pathways of glycerolipid biosynthesis in rat liver slices may be calculated from the results in Fig. 6 in the following manner:

$$\frac{d^{14}N_{\text{GP}}}{dt} = \frac{d^3N_{\text{GP}}}{dt} \cdot \frac{1}{R(t)} \quad (1)$$

where $^{14}N_{\text{GP}}$ = ^{14}C incorporation (d.p.m.) into lipid via the *sn*-glycerol 3-phosphate pathway, $^3N_{\text{GP}}$ = $2\text{-}^3\text{H}$ incorporation (d.p.m.) into lipid and $R = 2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in *sn*-glycerol 3-phosphate. Since all of these values vary with time it is necessary to integrate eqn. (1) from zero time of incubation to time T . Thus at time T :

$$\begin{aligned} ^{14}N_{\text{GP}}(T) - ^{14}N_{\text{GP}}(0) &= \int_0^T dt \frac{d^{14}N_{\text{GP}}(t)}{dt} \\ &= \int_0^T dt \frac{d^3N_{\text{GP}}(t)}{dt} \cdot \frac{1}{R(t)} \quad (2) \end{aligned}$$

Also:

$$\frac{d^{14}N_{\text{total}}}{dt} = \frac{d^{14}N_{\text{GP}}}{dt} + \frac{d^{14}N_{\text{DHAP}}}{dt} \quad (3)$$

where $^{14}N_{\text{total}}$ = total ^{14}C incorporation (d.p.m.) into lipid and $^{14}N_{\text{DHAP}}$ = ^{14}C incorporation (d.p.m.) into lipid via the dihydroxyacetone phosphate pathway. No correction has been made for a possible ^3H isotope effect of the acylation enzymes of the *sn*-glycerol 3-phosphate pathway. This seems to be justified, since the results in Figs. 1 and 2 indicate that there is little, if any, discrimination against $[1,3\text{-}^3\text{H}]\text{glycerol}$ compared with $[1\text{-}^{14}\text{C}]\text{glycerol}$ during lipid biosynthesis. Further, when isolated rat liver mitochondria were incubated with a mixture of *sn*- $[2\text{-}^3\text{H}]$ - and *sn*- $[1\text{-}^{14}\text{C}]\text{glycerol}$ 3-phosphate in the presence of antimycin A to prevent oxidation, the $^3\text{H}/^{14}\text{C}$ ratio in lipid was identical with that in the precursor *sn*-glycerol 3-phosphate (R. Manning & D. N. Brindley, unpublished work).

The rate of total ^{14}C incorporation into glycerolipid was constant at 4870 d.p.m./min per 100 mg wet wt. of liver throughout the experiment, whereas the rate of incorporation of $2\text{-}^3\text{H}$ comprised an initial lag phase followed, after 20 min, by a linear phase. This is

Fig. 7. Effect of incubation time on the relative importance of the dihydroxyacetone phosphate and glycerol phosphate pathways in the incorporation of glycerol into lipids by rat liver slices

The values are derived from the experiment illustrated in Fig. 6 after solving eqns. (2) and (3) (see the text). The rate of $[1\text{-}^{14}\text{C}]\text{glycerol}$ incorporation into lipid was constant over the period of incubation and was 4870 d.p.m./min per 100 mg wet wt. of tissue. The percentage of glycerol incorporated by the dihydroxyacetone phosphate pathway (----) and the glycerol phosphate pathway (—) is indicated. The absence of points on the curves is explained in the text.

reflected in the $2\text{-}^3\text{H}/1\text{-}^{14}\text{C}$ ratio in total glycerolipid (Fig. 6).

The ^{14}C contribution from the *sn*-glycerol 3-phosphate pathway at any time can be calculated from eqn. (2). The difference between this value and the total amount of ^{14}C in glycerolipid at that time is assumed to be due to incorporation of dihydroxy- $[1\text{-}^{14}\text{C}]\text{acetone}$ phosphate (eqn. 3). Therefore by solving eqns. (2) and (3) it is possible to construct a graph of the relative importance of the two pathways in rat liver slices (Fig. 7). All values substituted in eqn. (2) were derived from the curves drawn through experimental points from Fig. 6 and from the rates of incorporation of ^3H and ^{14}C , and are therefore average values. For this reason values of the relative proportions of the two pathways, derived from eqns. (2) and (3), are also average values and are therefore not represented by symbols in Fig. 7. However, substitution of the experimental values in eqn. (2) indicated that the relative quantitative importance of each pathway, shown in Fig. 7, is correct to $\pm 5\%$.

Initially most of the glycerol was incorporated via the glycerol phosphate pathway, but as oxidation of *sn*-glycerol 3-phosphate proceeded the contribution from the dihydroxyacetone phosphate pathway increased (Fig. 7). A steady-state position was attained

after 15 min of incubation, when the percentage of glycerol incorporated via the glycerol phosphate and dihydroxyacetone phosphate pathways was 40 and 60% respectively. Results from two further experiments indicated that, in the steady state, 40 and 50% respectively of the glycerol incorporated into glycerolipid proceeded via the glycerol phosphate pathway with the remainder being incorporated by the dihydroxyacetone phosphate pathway.

Discussion

In the present study the relative rates of incorporation for [$2\text{-}^3\text{H}$]glycerol and [$1\text{-}^{14}\text{C}$]glycerol into lipids by rat liver slices have been compared. The $^3\text{H}/^{14}\text{C}$ ratio in lipid rises owing to an isotope effect encountered when *sn*-glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate. Provided that the $^3\text{H}/^{14}\text{C}$ ratio in lipid is compared with that in glycerol phosphate, then values for the percentage of glycerol incorporated via the glycerol phosphate and dihydroxyacetone phosphate pathways can be obtained. However, when the ratio in lipid is compared with that in the glycerol (Hill & Lands, 1970; Okuyama & Lands, 1970) the results cannot be considered as a valid measurement for the two pathways.

The only other method that specifically attempts to give a quantitative measurement of the relative importance of the glycerol phosphate and dihydroxyacetone phosphate pathways is that of Agranoff & Hajra (1971). These investigators measured the relative incorporations of ^3H from ^3H -labelled NADH and ^3H -labelled NADPH into lipids of mouse liver and Ehrlich ascites-cell homogenates. They argued that conversion of dihydroxyacetone phosphate into *sn*-glycerol 3-phosphate would involve NADH, whereas acyl dihydroxyacetone phosphate reductase would specifically utilize NADPH. However, this method may also be subject to isotope effects, and, in addition, the acyldihydroxyacetone phosphate reductase may not be absolutely specific for NADPH (Rao *et al.*, 1970, 1971; Puleo *et al.*, 1970). A further disadvantage of this method is that it cannot be used with intact cells.

The method described in this paper can be used with subcellular fractions, tissue slices, isolated cells, whole organs or *in vivo*. The results obtained in the present study suggest that approximately equal percentages of glycerol pass through the glycerol phosphate and dihydroxyacetone phosphate pathways. This, however, may not represent the situation *in vivo*, since it is likely that the concentrations of *sn*-glycerol 3-phosphate, dihydroxyacetone phosphate, NAD^+ and NADH in liver slices are significantly different from those in the intact organ *in vivo* (Krebs & Veech, 1969). These changes could influence the flux of glycerol through the two pathways.

The values quoted in this paper for the relative importance of the glycerol phosphate and dihydroxyacetone phosphate pathways have been expressed in terms of the percentage of glycerol incorporated. No attempt was made to determine the specific radioactivities of the glycerol phosphate and dihydroxyacetone phosphate pools. If this analysis had been performed the molar synthesis of lipids from these two pools could have been determined. However, assuming that there is only one pool each of *sn*-glycerol 3-phosphate and dihydroxyacetone phosphate, the carbon source for the generation of the triose phosphates is irrelevant. Equivalent results would be obtained if it were possible to generate the specifically labelled precursors from glucose and fructose.

It is hoped that the method described in the present paper can be widely used to study the inter-relation and physiological significance of the glycerol phosphate and dihydroxyacetone phosphate pathways *in vivo*, which are at present unknown.

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