Heterogeneity of the *sn*-glycerol 3-phosphate pool in isolated hepatocytes, demonstrated by the use of deuterated glycerols and ethanol

Tomas CRONHOLM* and Tore CURSTEDT

Department of Physiological Chemistry, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

(Received 30 May 1984/Accepted 2 August 1984)

Hepatocytes were isolated from female rats and incubated with [1,1,3,3- $^2\text{H}_4$]glycerol or [2- ^2H]glycerol. The deuterium excess in phosphatidylcholines, *sn*-glycerol 3-phosphate and other organic acids was determined by g.l.c./mass spectrometry. The unlabelled fraction of the major phosphatidylcholines decreased exponentially, and the turnover was not changed by the presence of ethanol. The relative contribution of the two deuterated glycerols was about the same in the major phosphatidylcholine as in *sn*-glycerol 3-phosphate, indicating that formation by acylation of dihydroxyacetone phosphate is insignificant. [1,1,3,3- $^2\text{H}_4$]Glycerol had lost deuterium to a larger extent when it was incorporated in the phosphatidylcholine than when it was incorporated in *sn*-glycerol 3-phosphate, indicating that the phosphatidylcholines are formed from a separate pool of *sn*-glycerol 3-phosphate. Deuterium at C-2 was transferred between *sn*-glycerol 3-phosphate molecules to about 25%. Ethanol decreased the extent of deuterium transfer, the extent of glycerol uptake and the loss of deuterium at C-1 and C-3 in *sn*-glycerol 3-phosphate. The results indicate that the oxidation to dihydroxyacetone phosphate was inhibited by the NADH formed during ethanol oxidation. [2- ^2H]Glycerol also labelled an alcohol dehydrogenase substrate, malate and lactate, indicating oxidation of *sn*-glycerol 3-phosphate in the cytosol. The two acids appeared to be formed in reductions with different pools of NADH.

The mechanism behind the acute ethanol-induced fatty liver has been the subject of much investigation (Reitz, 1979). From these studies it has been concluded that both hepatic ethanol oxidation and increased peripheral lipolysis may be involved (Blomstrand & Forsell, 1971; Mallov & Bloch, 1956). Ethanol oxidation in the liver results in an increased concentration of *sn*-glycerol 3-phosphate (Reitz, 1979; Nikkilä & Ojala, 1963), and this might result in an increased formation of triacylglycerols and phosphoglycerides (Wirthensohn *et al.*, 1980; Debeer *et al.*, 1981). Results obtained during ethanol oxidation *in vivo* indicate that a separate pool of *sn*-glycerol 3-phosphate, mainly of gluconeogenic origin, is used for lipid biosynthesis (Curstedt & Sjövall, 1974). Since gluconeogenesis occurs mainly in the periportal region (Jungermann & Katz, 1982), it seems likely that this is also where the lipids are formed. Formation of phospholipids in the periportal cells

is also indicated by their secretion in bile together with bile acids (Gregory *et al.*, 1975), which pass through these cells during the enterohepatic circulation (Gumucio *et al.*, 1978). This functional zonation is absent in hepatocyte incubations, and it was therefore decided to study if the heterogeneity of *sn*-glycerol 3-phosphate was also absent. Preliminary results indicated that the approach used *in vivo*, involving the use of [1,1- $^2\text{H}_2$]ethanol to label newly formed molecules, could not be used to study the small amounts of endogenous *sn*-glycerol 3-phosphate and phosphatidylcholines which were formed in the short incubation period during which the hepatocytes remained metabolically intact. In the present study glycerols labelled with ^2H at C-1 and C-3 or at C-2 were instead used.

If peripheral lipolysis is increased during ethanol intoxication, there will be not only an increase in fatty acids but also an increased supply of glycerol to the liver. This might constitute an extra load on the hepatic redox state that is already deranged by ethanol. The oxidation of *sn*-glycerol

* To whom reprint requests should be addressed.

3-phosphate yields NADH in the cytosol or FADH₂ in mitochondria. The NADH might be used in various reductive processes, and it was considered that a study of the fate of the C-2 hydrogen of glycerol could provide additional information on the compartmentation of NADH in the liver cell (Cronholm & Curstedt, 1977; Blomberg & Cronholm, 1979). The metabolism of glycerol was studied by following the label in glycerol deuterated at C-1 and C-3 into pyruvate and tricarboxylic-acid-cycle acids.

Methods

Materials

[1,1-²H₂]Ethanol (96.7 atom% ²H) was obtained from Rohstoff-Einfuhr G.m.b.H., Düsseldorf, Germany. [2-²H]Glycerol was prepared from deuterated diethyl acetoxy malonate and LiAlH₄ as described by Hoberman & D'Adamo (1960). [1,1,3,3-²H₄]Glycerol was prepared by the same method from unlabelled diethyl acetoxy malonate and LiAl²H₄. The filtered aqueous solution was concentrated to 40 ml, and methanol (160 ml) was added. The mixture was percolated sequentially through a 30 g column of sulphopropyl-Sephadex (SP-Sephadex; Pharmacia Fine Chemicals, Uppsala, Sweden) (H⁺ form) and a 25 g column of Lipidex-DEAP (Packard Instrument Co., Downers Grove, IL, U.S.A.) (OH⁻ form), and eluted with about 200 ml of methanol/water (5:1, v/v). The solvents were removed under vacuum. The labelled glycerol was analysed as described below. In the [2-²H]glycerol the deuterium excess at C-2 was 93%, and the mean deuterium excess in the primary alcohol groups was below 1%. In the [1,1,3,3-²H₄]glycerol the mean deuterium excess of a hydrogen atom in the primary alcohol groups was 96.5%, and the deuterium excess at C-2 was below 1%.

Animals

Female Sprague-Dawley rats weighing 200–220 g were given food pellets (R3; Ewos, Södertälje, Sweden) and water *ad lib*. The animals were used at 08:30–10:00 h.

Preparation of isolated hepatocytes

The method used is a modification of that described by Berry & Friend (1969). Liver was perfused *in situ* with an oxygenated Ca²⁺-free buffer, followed by recycling an oxygenated buffer containing collagenase (type I; Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubation of the filtered cell suspension in the same buffer (Seglen, 1973). The hepatocytes were purified as described by Seglen (1973), except that the buffer used

throughout was the bicarbonate buffer described by Krebs & Henseleit (1932), containing bovine albumin (1.1 g/l; fraction V; Miles Laboratories, Elkhart, IN, U.S.A.) and 11 mM-glucose (Gibbons & Pullinger, 1977), equilibrated with O₂/CO₂ (19:1) and with pH adjusted to 7.4. At least 95% of the cells excluded Trypan Blue (Seglen, 1973). The wet weight was determined by centrifugation of 2 ml of cell suspension for 5 min at 1370 g and weighing after draining the tube upside down for 20 min. The volume of the hepatocyte suspension was adjusted to give 100 mg wet wt./2 ml. The yield from one liver was about 2.5 g wet wt., which corresponded to about 400 × 10⁶ cells.

Incubation procedure

The incubations were performed with 100 mg of cells in 4 ml of the Krebs–Henseleit bicarbonate buffer (pH 7.4) containing albumin and glucose as described above. When phosphorus-free organic acids were analysed, the cells were centrifuged and resuspended in fresh buffer immediately before the experiments. The incubation vials had a 7 cm² liquid surface area and there was a 10 ml/min flow of O₂/CO₂ (19:1) through the vials. These were placed in a rotary shaking bath (120 rev./min) at 37°C.

In the 2 h and 3 h experiments, substrates in 0.5 ml were added every 1 h. The initial concentration of ethanol was 20 mM in all experiments, and, by the addition of 40 μmol when glycerol was added, the concentration was kept above 10 mM as measured in some experiments at the end of the incubations by ethanol determination on 10 μl samples. The pH at the end of the incubations was about 7.4, and the fraction of cells that excluded Trypan Blue decreased to 80–85%. The incubations were stopped by addition of 1 ml of 3M-HClO₄ containing methoxyamine hydrochloride (10 mg/ml) when phosphorus-free organic acids were to be analysed, and by addition of 25 ml of methanol when *sn*-glycerol 3-phosphate, glycerol and phospholipids were to be analysed. In the former case, 0.4 ml of the incubation mixture was removed and added to 2.5 ml of methanol before addition of HClO₄. This sample was used for analysis of glycerol.

Isolation and analysis of metabolites

Phosphatidylcholines were isolated and analysed as previously described (Curstedt & Sjövall, 1974; Curstedt, 1974a, 1982). *sn*-Glycerol 3-phosphate was obtained from the polar layer in a Folch extraction and analysed as the trimethylsilyl derivative (Curstedt, 1974b). Lactic acid, β-hydroxybutyric acid and tricarboxylic-acid-cycle acids were isolated and analysed as previously described (Blomberg & Cronholm, 1979).

Cyclohexanol in the supernatant obtained by quenching incubations by addition of 1 ml of 3M-HClO₄, followed by neutralizing with 3M-KOH, was analysed by g.l.c./mass spectrometry as described by Cronholm & Fors (1976).

Glycerol was isolated from the aqueous methanol solution by sequential percolation through a column (120 mm × 4.4 mm) of SP-Sephadex (H⁺ form), and a column (120 mm × 4.4 mm) of Lipidex-DEAP (OH⁻ form), both packed in methanol/water (5:1, v/v). The sample was washed through the first column with 4 ml and the eluent was washed through the second column with 3 ml of methanol/water (5:1, v/v). The solvent was removed in vacuum, and the residue was treated with pyridine/hexamethyl-disilazane/trimethylchlorosilane (3:2:1, by vol.) for 15 min at room temperature. The trimethylsilyl ether was extracted with *n*-hexane and analysed by g.l.c. or g.l.c./mass spectrometry on packed SE-30 columns. G.l.c. quantification was performed with an electronic integrator and external standards.

Ethanol was determined by g.l.c. with propan-1-ol as internal standard on a column of 0.2% Carbowax 1500 on Carbowax C (Supelco, Bellefonte, PA, U.S.A.).

G.l.c./mass spectrometry was carried out with an LKB 9000 instrument by repetitive accelerating-voltage scanning and magnetic-tape recording as previously described (Axelson *et al.*, 1974). The abundances of molecules having different numbers of deuterium atoms were determined as previously described (Curstedt & Sjövall, 1974; Curstedt, 1974b; Blomberg & Cronholm, 1979). In the analysis of glycerol trimethylsilyl ether, *m/z* 293 (*M* - 15) was used to determine the deuterium content in the entire molecule, and *m/z* 103 to determine the deuterium content at C-1 and C-3.

Results

Uptake of labelled glycerol

The initial concentration of deuterated glycerol was 5.6 mM in most of the incubations. In order to maintain the glycerol concentration, labelled glycerol (15 μmol) was added after 1 h and 2 h. The final concentration in the 1 h incubation was only 0.1 mM in one experiment with 3.7 mM-glycerol (Expt. 5, Table 1), and the results from this experiment were therefore not used in the calculations of phosphatidylcholine turnover. In the other experiments, the final concentration varied between 0.8 mM and 11.5 mM. Thus the unphysiologically high glycerol concentration was chosen to ensure that labelled glycerol was present at all times. The uptake of glycerol during the 1 h incubations was 12.0 ± 3.7 μmol (S.D., *n* = 6) in the

absence of ethanol or when the oxidation was inhibited by 4-methylpyrazole. In the other experiments, i.e. during ethanol oxidation, this uptake was 6.8 ± 2.5 μmol (S.D., *n* = 5). The difference is significant (*P* < 0.05). The effect of ethanol oxidation was clearly seen in incubation of hepatocytes from one liver with ethanol in the absence or presence of 4-methylpyrazole. The uptake during 1 and 2 h incubations was 5.1 and 4.8 μmol respectively in the absence of inhibitor, and 12.9 and 22.8 μmol respectively in its presence.

Incorporation of [2-²H]glycerol

Hepatocytes from one rat were incubated with 3.7 mM-[2-²H]glycerol. The isolated free glycerol had about the same deuterium excess as the added [2-²H]glycerol. The *sn*-glycerol phosphate isolated after 1–3 h of incubation was mainly mono-deuterated (78–81%) and unlabelled. At least 90% of the ²H in the molecule was present at C-2 [calculated from the isotopic composition of the ions at *m/z* 328 (C-2) and 357 (C-2 + C-3) as described by Curstedt (1974b)]. The ²H excess was 90–92% of that in the added [2-²H]glycerol after 2 h and 3 h of incubation. The glycerol moiety of the phosphatidylcholines was labelled to an increasing extent during the 3 h of incubation. Most of the ²H (95%) in the labelled molecules of the major phosphatidylcholine, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, was present at C-2 [determined from the isotopic composition of the ions at *m/z* 103 and 112, as described by Curstedt & Sjövall (1974)].

Incorporation of [1,1,3,3-²H₄]glycerol

Incubation of hepatocytes with [1,1,3,3-²H₄]-glycerol resulted in labelling of *sn*-glycerol 3-phosphate and different molecular species of phosphatidylcholine. The isotopic composition of the molecules is shown in Table 1. The distribution of molecules labelled with different numbers of ²H atoms was the same in *sn*-glycerol 3-phosphate isolated after 1, 2 and 3 h of incubation. The fraction of unlabelled 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine molecules was much higher, which was assumed to be due to slow turnover. Comparisons between newly synthesized molecules were therefore made from the distributions among the deuterated molecules. In all cases the relative abundance of tetradeuterated molecules was lower, and that of dideuterated molecules higher, in the phosphatidylcholine than in the *sn*-glycerol 3-phosphate, indicating more extensive exchange of ²H for ¹H in the phosphatidylcholine precursor than in the analysed *sn*-glycerol 3-phosphate.

Table 1. *Isotopic composition of the glycerol moieties of sn-glycerol 3-phosphate (G3P) and of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PC) in hepatocytes incubated with [1,1,3,3- $^2\text{H}_4$]glycerol*

The initial total concentration of labelled glycerol was 3.7 mM in Expt. 5 and 5.6 mM in all other experiments. The initial concentrations of [2- ^2H]- and [1,1,3,3- $^2\text{H}_4$]glycerol were approximately equal. The values are mean values from duplicate incubations. Each experiment was carried out with hepatocytes from one liver.

Expt. no.	Addition to incubation	Compound	Time of incubation (h)	Percentage of unlabelled molecules	Percentage distribution of labelled molecules					Percentage of molecules labelled at C-2+C-3		
					$^2\text{H}_1$	$^2\text{H}_2$	$^2\text{H}_3$	$^2\text{H}_4$	$^2\text{H}_5$	$^2\text{H}_1$	$^2\text{H}_2$	$^2\text{H}_3$
2	—	G3P	1	22.0	2.8	19.0	16.9	60.9	0.5	4.8	71.7	0.9
		G3P	2	8.6	2.5	16.3	15.4	65.6	0.2	5.2	81.5	0.8
		G3P	3	7.0	2.2	13.8	13.6	70.3	0.1	5.3	85.4	0.6
		PC	3	67.2	6.3	25.4	16.0	52.3	0.0	—	—	—
3	—	G3P	1	11.7	4.1	26.2	19.0	50.1	0.6	7.2	77.6	1.9
		G3P	2	13.4	4.5	29.0	19.1	46.7	0.7	7.2	76.1	1.9
		G3P	3	13.7	4.5	28.6	18.5	47.5	0.9	7.1	75.3	2.0
		PC	3	56.3	5.8	30.1	19.3	43.2	1.6	—	—	—
4	[2- ^2H]Glycerol	G3P	1	13.7	50.3	5.2	10.3	31.8	2.4	43.3	30.0	10.6
		G3P	2	11.0	51.0	4.3	9.3	33.2	2.1	46.8	30.3	10.2
		G3P	3	9.9	50.8	4.0	8.7	34.5	2.0	47.7	31.4	9.5
		PC	3	66.5	46.0	10.4	10.8	30.1	2.8	—	—	—
5	[2- ^2H]Glycerol	G3P	1	64.9	60.1	9.6	9.4	19.0	1.9	20.4	8.8	3.4
		G3P	2	24.8	55.9	9.0	11.6	21.0	2.5	42.0	20.2	10.1
		G3P	3	14.0	58.6	5.6	9.9	23.5	2.4	51.1	20.5	10.9
		PC	3	66.8	51.9	13.6	11.2	20.4	3.0	—	—	—
4	20 mM-[1,1- $^2\text{H}_2$]-Ethanol	G3P	1	4.7	3.9	11.1	15.1	69.0	0.9	8.3	77.5	7.2
		G3P	2	4.2	3.0	8.8	12.4	75.3	0.5	6.6	81.7	5.7
		G3P	3	3.9	2.7	7.4	10.8	78.8	0.3	6.4	82.5	4.9
		PC	3	68.5	5.6	14.3	12.9	64.2	2.9	—	—	—
6	20 mM-[1,1- $^2\text{H}_2$]-Ethanol	G3P	1	5.5	3.7	14.2	17.3	63.2	1.6	5.3	79.1	8.9
		G3P	2	4.8	3.5	13.2	15.8	66.2	1.3	5.2	80.8	7.7
		G3P	3	4.7	3.6	11.6	14.5	69.0	1.5	5.9	79.7	8.3
		PC	3	67.3	6.7	16.0	15.3	56.9	5.0	—	—	—
7	20 mM-Ethanol	G3P	1	7.4	2.7	15.0	15.4	66.4	0.5	5.1	84.0	1.3
		G3P	2	7.2	2.5	13.1	13.0	71.0	0.4	5.0	84.7	1.3
		G3P	3	6.6	2.6	11.9	11.7	73.4	0.4	5.1	85.4	1.3
		PC	3	63.5	4.6	17.2	13.1	63.8	1.2	—	—	—
8	20 mM-Ethanol	G3P	1	5.8	2.2	14.2	13.8	68.9	0.9	4.7	84.8	1.6
		G3P	2	5.9	2.1	12.0	11.6	73.4	0.9	4.7	85.9	1.6
		G3P	3	5.8	2.2	10.9	10.1	75.5	1.2	4.8	86.0	1.6
		PC	3	82.6	3.5	17.7	13.9	63.2	1.6	—	—	—
8	20 mM-Ethanol + 1.7 mM-4-methylpyrazole	G3P	1	22.4	3.7	23.4	15.8	56.3	0.8	5.5	78.8	1.8
		G3P	2	14.1	3.1	20.5	15.1	60.7	0.5	5.0	80.6	1.4
		G3P	3	12.3	3.6	24.1	15.6	55.8	0.9	5.2	80.7	1.7
		PC	3	53.1	5.4	27.3	16.9	47.6	2.8	—	—	—
9	[2- ^2H]Glycerol + 20 mM-ethanol	G3P	1	13.4	53.8	5.0	7.0	33.3	0.8	48.3	33.4	3.5
		G3P	2	12.4	53.6	4.5	6.2	35.0	0.7	48.8	34.9	2.7
		G3P	3	11.8	53.6	4.3	5.7	35.8	0.6	49.0	36.1	2.2
		PC	3	69.6	51.4	7.4	6.6	32.9	1.8	—	—	—

Incorporation of a mixture of [1,1,3,3- $^2\text{H}_4$]glycerol and [2- ^2H]glycerol

Incubation of hepatocytes with a mixture of the deuterated glycerols in approximately equal concentrations resulted in the expected increase of monodeuterated *sn*-glycerol 3-phosphate molecules above that obtained with [1,1,3,3- $^2\text{H}_4$]glycerol alone (Table 1). The relative abundance of tetra-

deuterated molecules was lower and that of dideuterated molecules higher among labelled phosphatidylcholine molecules than among labelled *sn*-glycerol 3-phosphate molecules. Examination of the isotopic composition of the C-2+C-3 part of the *sn*-glycerol 3-phosphate molecule revealed the presence of molecules labelled at both C-2 and C-3, which were only

present in trace amounts when [1,1,3,3-²H₄]-glycerol was incubated alone. This indicates that deuterium was transferred from *sn*-glycerol 3-phosphate labelled at C-2 to *sn*-glycerol 3-phosphate labelled at C-1 and C-3. It was calculated that about 25% of the latter molecules had received a ²H atom. The ratio between the abundances of penta- and tetra-deuterated phosphatidylcholine molecules was higher in the experiment with both deuterated glycerols than in the experiment with only [1,1,3,3-²H₄]-glycerol, indicating that the precursor of the glycerol moiety had also received ²H by transfer from C-2.

The relative abundance of monodeuterated molecules was slightly lower for the phosphatidylcholine than for *sn*-glycerol 3-phosphate, although the opposite was true when only [1,1,3,3-²H₄]-glycerol was incubated. Thus the glycerol residue in phosphatidylcholine was slightly less deuterated at C-2 than was *sn*-glycerol 3-phosphate.

Incorporation of deuterated glycerols in the presence of ethanol and [1,1-²H₂]ethanol

The abundance of unlabelled *sn*-glycerol 3-phosphate in incubations with [1,1,3,3-²H₄]-glycerol was lower during oxidation of ethanol or [1,1-²H₂]ethanol (Table 1). The effect was most clearly seen when hepatocytes from one liver were incubated with ethanol in the absence or presence of 4-methylpyrazole. In both *sn*-glycerol 3-phosphate and the phosphatidylcholine the labelled molecules were tetradeuterated to a larger extent and dideuterated to a smaller extent during oxidation of unlabelled or labelled ethanol than

otherwise. Ethanol oxidation caused practically no decrease in the ²H exchange in the C-2+C-3 part of *sn*-glycerol 3-phosphate. Thus, at least in this compound, the effect was mainly due to a decreased ²H exchange at C-1.

When hepatocytes were incubated with an approximately equimolar mixture of [2-²H]glycerol and [1,1,3,3-²H₄]-glycerol in the presence of ethanol, the abundance of molecules trideuterated at C-2+C-3 was not much higher than in the incubations of [1,1,3,3-²H₄]-glycerol alone. Thus ethanol oxidation inhibited the transfer of ²H at C-2 between glycerol molecules. The presence of ethanol did not change the relative utilization of the differently labelled glycerols in the synthesis of *sn*-glycerol 3-phosphate or the phosphatidylcholine.

Incubation of hepatocytes with [1,1,3,3-²H₄]-glycerol and [1,1-²H₂]ethanol resulted in the appearance of *sn*-glycerol 3-phosphate molecules that were trideuterated at C-2+C-3. About 5–10% of the labelled molecules appeared to have ²H at C-2.

In all incubations of hepatocytes with labelled glycerols and labelled or unlabelled ethanol, the abundance of tetradeuterated molecules was lower and that of dideuterated molecules was higher among the labelled molecules of the phosphatidylcholine than among the labelled molecules of *sn*-glycerol 3-phosphate, as was also observed in the absence of ethanol.

Turnover of phosphatidylcholines

The species composition of the phosphatidylcholines was analysed in an experiment with [2-

Table 2. Apparent half-lives of phosphatidylcholines in hepatocytes incubated with deuterated glycerols
The values were calculated from the decrease in the unlabelled fraction, assuming a single pool and labelling of all newly synthesized molecules. The results shown are the ranges obtained from the 1, 2 and 3 h values. Primary values were means from duplicate incubations. Each experiment was carried out with hepatocytes from one liver.

Expt. no.	Substrates and additions	Apparent half-life (h) of phosphatidylcholines with acyl group at C-1/acyl group at C-2			
		C _{16:0} /C _{18:2}	C _{16:0} /C _{18:1}	C _{18:0} /C _{18:2}	C _{16:0} /C _{16:0}
1	5.6 mM-[2- ² H]Glycerol	4.6–5.2	3.3–3.6	22.7–34.4	1.9–3.4
2	5.6 mM-[1,1,3,3- ² H ₄]Glycerol	4.7–5.2	4.4–4.6	14.6–16.5	2.6–5.2
3	5.6 mM-[1,1,3,3- ² H ₄]Glycerol	4.0–4.4	3.4–3.6	13.8–15.3	–
4	2.8 mM-[2- ² H]Glycerol + 2.8 mM-[1,1,3,3- ² H ₄]glycerol	4.7–5.2	4.6–5.4	19.4–24.9	–
4	5.6 mM-[1,1,3,3- ² H ₄]Glycerol + 20 mM-[1,1- ² H ₂]ethanol	4.7–5.5	5.5–5.9	17.7–20.8	2.5–4.8
6	5.6 mM-[1,1,3,3- ² H ₄]Glycerol + 20 mM-[1,1- ² H ₂]ethanol	4.9–5.3	4.9–5.2	17.6–19.2	–
7	5.6 mM-[1,1,3,3- ² H ₄]Glycerol + 20 mM-ethanol	4.3–4.6	4.4–4.6	15.4–16.9	–
8	5.6 mM-[1,1,3,3- ² H ₄]Glycerol + 20 mM-ethanol	3.6–4.0	3.4–3.6	15.5–35.9	–
8	5.6 mM-[1,1,3,3- ² H ₄]Glycerol + 20 mM-ethanol + 1.7 mM-4-methylpyrazole	2.9–3.3	2.4–2.6	12.8–15.3	–
9	2.8 mM-[2- ² H]Glycerol + 2.8 mM-[1,1,3,3- ² H ₄]glycerol + 20 mM-ethanol	4.6–5.7	4.4–5.1	14.7–17.1	–

Table 3. *Incorporation of deuterium in hepatocytes incubated with 5.6 mM-[2-²H]glycerol*
 Values are means $\pm$ S.D. from four parallel incubations with hepatocytes from one liver in each experiment.

Expt. no.	Addition to incubation	Compound	Percentage of labelled molecules at incubation time			
			15 min		60 min	
			² H ₁	² H ₂	² H ₁	² H ₂
10	–	Lactate	4.7 $\pm$ 0.4	0.0 $\pm$ 0.0	19.5 $\pm$ 0.6	0.2 $\pm$ 0.0
		β -Hydroxybutyrate	0.6 $\pm$ 0.2	0.1 $\pm$ 0.1	1.6 $\pm$ 0.1	0.1 $\pm$ 0.0
		Fumarate	10.6 $\pm$ 0.9	0.3 $\pm$ 0.4	16.2 $\pm$ 1.2	0.5 $\pm$ 0.1
		Malate	10.3 $\pm$ 0.5	0.6 $\pm$ 0.2	18.2 $\pm$ 0.8	1.7 $\pm$ 0.3
11	20 mM-Ethanol	Lactate	1.5 $\pm$ 1.2	0.0 $\pm$ 0.0	2.6 $\pm$ 0.3	0.0 $\pm$ 0.0
		β -Hydroxybutyrate	0.2 $\pm$ 0.3	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0
		Fumarate	3.6 $\pm$ 0.6	0.8 $\pm$ 0.2	3.6 $\pm$ 1.4	0.2 $\pm$ 0.2
		Malate	2.7 $\pm$ 0.3	0.2 $\pm$ 0.1	2.8 $\pm$ 0.5	0.5 $\pm$ 0.4
12	0.25 mM-Cyclohexanone	Cyclohexanol	12.4 $\pm$ 3.4	–	20.1 $\pm$ 2.3	–

Table 4. *Labelling of organic acids in hepatocytes incubated with 5.6 mM-[1,1,3,3-²H₄]glycerol*
 Values are means $\pm$ S.D. from four parallel incubations with hepatocytes from one liver.

Compound	Percentage of labelled molecules at incubation time					
	15 min			60 min		
	² H ₁	² H ₂	² H ₃	² H ₁	² H ₂	² H ₃
Pyruvate	7.8 $\pm$ 1.7	8.7 $\pm$ 4.6	0.0 $\pm$ 0.0	9.6 $\pm$ 2.9	15.1 $\pm$ 3.8	0.0 $\pm$ 0.0
Fumarate	10.9 $\pm$ 0.8	1.6 $\pm$ 0.1	–	15.0 $\pm$ 3.3	2.1 $\pm$ 0.9	–
Malate	10.2 $\pm$ 0.5	0.9 $\pm$ 0.3	–	9.7 $\pm$ 0.2	0.8 $\pm$ 0.2	–
α -Oxoglutarate*	0.1 $\pm$ 0.2	0.9 $\pm$ 0.4	0.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.6 $\pm$ 0.2	0.0 $\pm$ 0.0
Citrate	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.2 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

* The mass-spectrometric fragment used has lost one of the methylene hydrogen atoms (Blomberg & Cronholm, 1979).

²H]glycerol (Expt. 1, Table 2) by a previously described method (Curstedt, 1982), and was the same as found in that study for liver *in vivo*, with no changes during the incubation. When hepatocytes were incubated with labelled glycerol, the fraction of unlabelled molecules of the major species decreased in a way consistent with first-order kinetics. The apparent half-lives were calculated from the unlabelled fraction remaining at different times (Table 2). There were no consistent differences between results obtained in the presence or absence of ethanol oxidation. The decrease in the unlabelled fraction of the minor species 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine reached a plateau after the first 1 h, and the divergence from first-order kinetics is apparent from the wide range in calculated half-lives.

Transfer of ²H from [2-²H]glycerol

The ²H from [2-²H]glycerol was incorporated in lactate, β -hydroxybutyrate, fumarate and malate (Table 3). The abundance of monodeuterated molecules of these acids was higher after 60 min

than after 15 min of incubation, and lower when ethanol was present in the incubations. The abundance of dideuterated molecules of lactate was very low, indicating that only one position was significantly labelled. The isotopic compositions of fumarate and malate were consistent with the expected presence of two equally labelled positions. Thus the results indicate that the ²H excess in one position of lactate was about twice that of one position in malate or fumarate.

When cyclohexanone was incubated with hepatocytes in the presence of [2-²H]glycerol, the cyclohexanol produced was labelled, and the ²H excess was higher after 60 min than after 15 min (Table 3).

Incorporation of [1,1,3,3-²H₄]glycerol in organic acids

Pyruvate became both mono- and di-deuterated, whereas fumarate and malate became essentially only monodeuterated, when hepatocytes were incubated with [1,1,3,3-²H₄]glycerol (Table 4). Under the same conditions, α -oxoglutarate and citrate remained unlabelled.

Discussion

Turnover of phosphatidylcholines

For the three major phosphatidylcholine species, the unlabelled fraction decreased exponentially during the incubations with deuterated glycerols, indicating the presence of only one pool. The half-lives have previously been determined *in vivo* by using [1,1- $^2\text{H}_2$]ethanol to label the newly formed molecules (Curstedt, 1974a). The half-lives for 1-palmitoyl-2-linoleoyl- and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine were about twice as long as *in vivo*, and the half-life of 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine was about 5 times longer than *in vivo*. These differences were not due to the presence of ethanol in the experiments *in vivo*, since addition of ethanol did not change the turnover in the hepatocyte incubations. The difference could be due to a less efficient supply of necessary fatty acids than *in vivo*, since it is known that the composition of the fatty acids bound to albumin affects the composition of the newly formed phosphatidylcholines (Sundler *et al.*, 1974). However, the results are in general agreement with the observation that biosynthesis of glycerolipids in isolated hepatocytes proceeds essentially as *in vivo* (Ontko, 1972; Sundler *et al.*, 1974; Groener & van Golde, 1977).

The decrease in the fraction of unlabelled 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was more rapid during the first hour than in the second and third hours of incubation. This might be due to the presence of two pools or to the utilization of an incompletely labelled precursor for the glycerol moiety. With the few time-points obtained, it was not possible to determine the half-lives accurately, but apparently the initial half-life was close to that seen in bile *in vivo* (1 h), but clearly different from that in liver (12 h) or plasma (17 h) (Curstedt, 1974a). Under conditions *in vivo*, the major hepatic pool of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine is probably in rapid equilibrium with plasma and extrahepatic pools with slow turnover, whereas a small hepatic pool with a short half-life is used for biliary secretion.

Metabolism of glycerol and *sn*-glycerol 3-phosphate

The uptake of glycerol was 12 $\mu\text{mol/h}$. This is in agreement with earlier investigations (Berry *et al.*, 1973; Schimassek *et al.*, 1974). The uptake was inhibited by 20 mM-ethanol, and the inhibition of *sn*-glycerol 3-phosphate oxidation was also seen from the decreases in the loss of ^2H by exchange in the glycerol moieties and of the abundance of unlabelled glycerol moieties. ^2H from [1,1- $^2\text{H}_2$]ethanol was incorporated at C-2 in *sn*-glycerol 3-phosphate, and unlabelled ethanol decreased the intermolecular transfer of the C-2 hydrogen

between *sn*-glycerol 3-phosphate molecules. Thus it seems likely that the mechanism is the same as suggested for the inhibition produced by sorbitol, i.e. accumulation of cytosolic NADH (Berry *et al.*, 1973). ^2H from [2- ^2H]glycerol was incorporated in cyclohexanol that was formed in a reaction catalysed by alcohol dehydrogenase, giving further support to the concept of interaction via a shared NADH pool. The NADH-dependent accumulation of *sn*-glycerol 3-phosphate could result in inhibition of glycerokinase, which is not normally rate-limiting. It might be speculated that this would increase the relative importance of the kidneys for gluconeogenesis during ethanol oxidation.

The ^2H excess at C-2 in *sn*-glycerol 3-phosphate was about 90% of that in the added [2- ^2H]glycerol. This decrease could have been due to supply of endogenous substrates from lipolysis or intermediates of the glycolytic or gluconeogenic pathways, and it could have been due to exchange in reversible oxidoreduction of *sn*-glycerol 3-phosphate. Thus the observation that ^2H was transferred from [2- ^2H]glycerol to 25% of simultaneously metabolized [1,1,3,3- $^2\text{H}_4$]glycerol shows that rapid oxidoreduction took place. The appearance of dideuterated molecules of *sn*-glycerol 3-phosphate in incubations with [1,1,3,3- $^2\text{H}_4$]glycerol indicates that it was reversibly converted in the sequence dihydroxyacetone phosphate $\rightarrow$ glyceraldehyde 3-phosphate $\rightarrow$ 3-phosphoglycerate or fructose 1,6-bisphosphate. That the loss of ^2H took place mainly at C-1 is shown by the comparatively high retention in the C-2 + C-3 part of the molecule. The abundance of dideuterated molecules was higher than that of trideuterated molecules, indicating that a molecule that had lost one ^2H atom in the sequence often also had lost one more. This indicates that the slow step in the equilibration was the oxidation to dihydroxyacetone phosphate.

When pyruvate is formed from *sn*-glycerol 3-phosphate in the glycolytic pathway, the hydrogen atoms at C-3 are not expected to exchange with water. However, the ratio between mono- and dideuterated molecules was higher in pyruvate than in the C-2 + C-3 part of *sn*-glycerol 3-phosphate in incubations with [1,1,3,3- $^2\text{H}_4$]glycerol. This exchange at C-3 of pyruvate could have been due to reversible formation of glucose 6-phosphate or to the 'futile cycle' involving oxaloacetate, malate and phosphoenolpyruvate (Hue, 1982). Thus malate was monodeuterated to 10%, but not dideuterated, and could have contributed to monodeuterated pyruvate via extramitochondrial oxaloacetate. The absence of dideuterated malate molecules indicates rapid interconversion of fumarate and malate, also seen from the similar labelling of these acids. The abundance of monodeuterated molecules was close

to that expected from formation from the analysed pyruvate. This indicates that the malate-oxaloacetate interconversion was slow in comparison with the net formation of malate from pyruvate, and thus that the malate dehydrogenase reactions were not close to equilibrium. Rapid reversible reactions in the sequence oxaloacetate→malate→fumarate would have caused loss of ^2H from these acids.

Citrate and α -oxoglutarate remained unlabelled during metabolism of $[1,1,3,3\text{-}^2\text{H}_4]$ glycerol, although intramitochondrial oxaloacetate presumably was labelled as discussed above. Thus the forward rate in the tricarboxylic acid cycle was apparently low, and it seems likely that this inhibition was due to the NADH formed during metabolism of the added glycerol and thus similar to the effect of ethanol metabolism.

^2H removed in the oxidation of *sn*- $[2\text{-}^2\text{H}]$ glycerol 3-phosphate could label cytosolic NADH, indicating that the oxidation was not only catalysed by the mitochondrial enzyme. Thus lactate, fumarate and malate became labelled when hepatocytes were incubated with $[2\text{-}^2\text{H}]$ glycerol. The labelling of the NADH utilized in the lactate dehydrogenase reaction appeared to be about twice that of the NADH utilized in the malate dehydrogenase reaction. A difference between the NADH pools used by these two enzymes has previously been observed *in vivo* (Blomberg & Cronholm, 1979).

The increase in ^2H excess in the organic acids formed during metabolism of $[2\text{-}^2\text{H}]$ - and $[1,1,3,3\text{-}^2\text{H}_4]$ glycerol probably reflects accumulation of *sn*-glycerol 3-phosphate (Berry *et al.*, 1973). The ^2H excess of the NADH used by alcohol dehydrogenase also increased, as shown by the increasing labelling of cyclohexanol in the presence of $[2\text{-}^2\text{H}]$ glycerol. The ^2H excess in the acids was lower in the presence of ethanol (Table 3). This effect was more pronounced than expected from the labelling of the acids in incubations of hepatocytes with (1*R*) $[1\text{-}^2\text{H}]$ - and (1*S*) $[1\text{-}^2\text{H}]$ -ethanol (Cronholm, 1983), indicating that it was due not only to dilution from NADH formed in the oxidation of alcohol but also to inhibition of the oxidation of *sn*-glycerol 3-phosphate in the presence of ethanol, as discussed above.

Precursors of the glycerol moiety of phosphatidylcholines

Phosphatidic acids are formed by two different pathways, utilizing *sn*-glycerol 3-phosphate (Kennedy, 1953; Kornberg & Pricer, 1953) or dihydroxyacetone phosphate (Hajra, 1968) in the acylation reaction. It has also been observed that, at least during ethanol oxidation *in vivo*, the *sn*-glycerol phosphate pool used in the formation of phosphatidic acids is not equilibrated with

the major hepatic *sn*-glycerol 3-phosphate pool (Curstedt & Sjövall, 1974; Curstedt, 1974a). These aspects of phosphatidylcholine biosynthesis were discussed in a review by Cronholm *et al.* (1982). The present experiments were designed to provide further information on these questions, especially with regard to the effects of ethanol on lipid biosynthesis. The results indicated that also in isolated hepatocytes and in both the absence and the presence of ethanol oxidation the glycerol moiety of phosphatidylcholine was derived from a pool of hepatic *sn*-glycerol 3-phosphate that was separate from the bulk pool. Thus in incubations with $[1,1,3,3\text{-}^2\text{H}_4]$ glycerol the exchange of ^2H for ^1H was more extensive in the glycerol moiety of the phosphatidylcholine than in the *sn*-glycerol 3-phosphate. This shows that the latter was not a precursor of the former, but the result does not distinguish between compartmentation of the *sn*-glycerol 3-phosphate pool and formation of phosphatidylcholine from dihydroxyacetone phosphate. Since oxidation to dihydroxyacetone phosphate is necessary for the exchange, it can be concluded that the phosphatidylcholine precursor had undergone this oxidation to a larger extent than the major pool of *sn*-glycerol 3-phosphate.

The intermolecular ^2H transfer at C-2 of *sn*-glycerol 3-phosphate observed in incubations with $[1,1,3,3\text{-}^2\text{H}_4]$ - and $[2\text{-}^2\text{H}]$ -glycerol indicates that molecules that had been oxidized to dihydroxyacetone phosphate were again reduced to a large extent. The relative abundance of molecules labelled with different numbers of deuterium atoms indicated that this was also true of the glycerol moiety in the phosphatidylcholine. This would have been unlikely if acylated dihydroxyacetone phosphate was a precursor. The NADPH used in the reduction of this precursor (Hajra, 1968) would have been less labelled than the NADH bound to glycerol 3-phosphate dehydrogenase. Citrate and malate might transfer ^2H from NADH to NADPH. The absence of labelling of citrate and low labelling of malate indicate considerable dilution of the label during this transfer. Furthermore, in these incubations the abundance of monodeuterated newly synthesized phosphatidylcholine molecules was not much lower than the abundance of monodeuterated molecules among the labelled *sn*-glycerol 3-phosphate molecules. The difference that was actually observed could be completely explained by the more extensive oxidoreduction of the phosphatidylcholine precursor that was discussed above.

Thus the results are not consistent with any significant formation of the phosphatidylcholine via acylation of dihydroxyacetone phosphate. Consequently, they strongly support the conten-

tion that the *sn*-glycerol 3-phosphate pool is heterogeneous (Curstedt & Sjövall, 1974). Thus the heterogeneity of the hepatic *sn*-glycerol 3-phosphate pool was not due to functional zonation in the liver lobule caused, e.g., by intralobular differences in oxygenation or bile acid excretion (Gumucio *et al.*, 1978). It is therefore concluded that the heterogeneity is due to enzymic differences between the cells or to subcellular compartmentation. The ^2H incorporation in organic acids from C-2 of $[2\text{-}^2\text{H}]$ glycerol and at C-2 in *sn*-glycerol 3-phosphate from $[1,1\text{-}^2\text{H}_2]$ ethanol support the suggestion that transfer of labelled hydrogen via NADH and other substrates and back to *sn*-glycerol 3-phosphate might explain the rising $^3\text{H}/^{14}\text{C}$ ratio of *sn*-glycerol 3-phosphate in incubations with $[\text{U-}^{14}\text{C}, 2\text{-}^3\text{H}]$ glycerol (Okuyama & Lands, 1970; Manning & Brindley, 1972; Rognstad *et al.*, 1974) that has been used as evidence in favour of the presence of a dihydroxyacetone phosphate pathway (Manning & Brindley, 1972).

The skilful technical assistance of Britt Andrée, Birgitta Berthold, Monica Bredmyr, Gudrun Green and Margareta Viestam-Rains is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (Grant No. 25X-2189), Karolinska Institutet and Magnus Bergvalls Stiftelse.

References

- Axelson, M., Cronholm, T., Curstedt, T., Reimendal, R. & Sjövall, J. (1974) *Chromatographia* **7**, 502–509
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Berry, M. N., Kun, E. & Werner, H. V. (1973) *Eur. J. Biochem.* **33**, 407–417
- Blomberg, S. & Cronholm, T. (1979) *Eur. J. Biochem.* **101**, 111–117
- Blomstrand, R. & Forsell, L. (1971) *Life Sci.* **10**, 523–530
- Cronholm, T. (1983) *Pharmacol. Biochem. Behav.* **18**, Suppl. 1, 229–232
- Cronholm, T. & Fors, C. (1976) *Eur. J. Biochem.* **70**, 83–87
- Cronholm, T. & Curstedt, T. (1977) *Eur. J. Biochem.* **77**, 337–340
- Cronholm, T., Curstedt, T. & Sjövall, J. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 331–359, Academic Press, London
- Curstedt, T. (1974a) *Biochim. Biophys. Acta* **369**, 196–208
- Curstedt, T. (1974b) *Eur. J. Biochem.* **49**, 355–359
- Curstedt, T. (1982) *Biochim. Biophys. Acta* **713**, 589–601
- Curstedt, T. & Sjövall, J. (1974) *Biochim. Biophys. Acta* **369**, 173–195
- Debeer, L. J., Declercq, P. E. & Mannaerts, G. P. (1981) *FEBS Lett.* **124**, 31–34
- Gibbons, G. F. & Pullinger, C. R. (1977) *Biochem. J.* **161**, 321–330
- Gregory, D. H., Vlahcevic, Z. R., Schatzki, P. & Swell, L. (1975) *J. Clin. Invest.* **55**, 105–114
- Groener, J. E. M. & van Golde, L. M. G. (1977) *Biochim. Biophys. Acta* **487**, 105–114
- Gumucio, J. J., Balabaud, C., Miller, D. L., DeMason, L. J., Appelman, H. D., Stoecker, T. J. & Franzblau, D. R. (1978) *J. Lab. Clin. Med.* **91**, 350–362
- Hajra, A. K. (1968) *J. Biol. Chem.* **243**, 3458–3465
- Hoberman, H. D. & D'Adamo, A., Jr. (1960) *J. Biol. Chem.* **235**, 1599–1602
- Hue, L. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 71–97, Academic Press, London
- Jungermann, K. & Katz, N. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 411–435, Academic Press, London
- Kennedy, E. P. (1953) *J. Biol. Chem.* **201**, 399–412
- Kornberg, A. & Pricer, W. E., Jr. (1953) *J. Biol. Chem.* **204**, 345–357
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Mallov, S. & Bloch, J. L. (1956) *Am. J. Physiol.* **184**, 29–34
- Manning, R. & Brindley, D. N. (1972) *Biochem. J.* **130**, 1003–1012
- Nikkilä, E. A. & Ojala, K. (1963) *Proc. Soc. Exp. Biol. Med.* **113**, 814–817
- Okuyama, H. & Lands, W. E. M. (1970) *Biochim. Biophys. Acta* **218**, 376–377
- Ontko, J. A. (1972) *J. Biol. Chem.* **247**, 1788–1800
- Reitz, R. C. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E. & Noble, P., eds.), vol. 1, 353–382, Plenum Press, New York and London
- Rognstad, R., Clark, D. G. & Katz, J. (1974) *Biochem. J.* **140**, 249–251
- Schimassek, H., Walli, A. K., Ferraudi, M. & Jost, U. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F., ed.), pp. 715–725, Munksgaard, Copenhagen
- Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398
- Sundler, R., Åkesson, B. & Nilsson, Å. (1974) *J. Biol. Chem.* **249**, 5102–5107
- Wirthensohn, G., Brocks, D. G. & Guder, W. G. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 985–993