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Glyceroneogenesis and the Source of Glycerol for Hepatic Triacylglycerol Synthesis in Humans*

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Glyceroneogenesis, *i.e.* the synthesis of the glycerol moiety of triacylglycerol from pyruvate, has been suggested to be quantitatively important in both the liver and adipose tissue during fasting. However, the actual contribution of glyceroneogenesis to triacylglycerol synthesis has not been quantified *in vivo* in human studies. In the present study we have measured the contribution of glycerol and pyruvate to *in vivo* synthesis of hepatic triacylglycerol in nonpregnant and pregnant women after an overnight fast. Five nonpregnant women were administered [$^{13}C_3$]glycerol tracer as prime constant rate infusion, and the appearance of tracer in plasma glucose and triacylglycerol was quantified using gas chromatography-mass spectrometry. The contribution of pyruvate to hepatic triacylglycerol was quantified in nonpregnant and pregnant women using the deuterium labeling of body water method. The appearance of [^{2}H] in hydrogens on C_1 and C_3 of triacylglycerol was measured following periodate oxidation of the glycerol isolated from hydrolyzed triacylglycerol. After a 16-h fast, ~6.1% of the plasma triacylglycerol pool was derived from plasma glycerol, whereas 10 to 60% was derived from pyruvate in nonpregnant women and pregnant women early in gestation. Our data suggest that glyceroneogenesis from pyruvate is quantitatively a major contributor to plasma triacylglycerol synthesis and may be important for the regulation of very low density lipoprotein triacylglycerol production. Our data also suggest that 3-glycerol phosphate is in rapid equilibrium with the triosephosphate pool, resulting in rapid labeling of the triose pool by the administered tracer glycerol. Because the rate of flux of triosephosphate to glucose during fasting far exceeds that to triacylglycerol, more glycerol ends up in glucose than in triacylglycerol. Alternatively, there may be two distinct pools of 3-glycerol phosphate in the liver, one involved in generating triosephosphate from glycerol and the other involved in glyceride-glycerol synthesis.

The synthesis of triacylglycerol in the liver, adipose tissue, and skeletal muscle following a meal is an important metabolic pathway for the deposition of fat and in the maintenance of energy homeostasis in all vertebrates. Even after an overnight fast in adult humans, and following a brief fast in newborn infants, a substantial re-esterification of fatty acids has been documented using isotopic tracer methods (1–3). The source of glycerol for the esterification of fatty acids in various tissues has generally been considered to be plasma glucose or glycerol; however direct evidence for such an inference has not been documented.

Triacylglycerol synthesis requires both fatty acids and a source of 3-glycerol phosphate. During fasting, the source of 3-glycerol phosphate can either be plasma glucose via glycolysis or glycerol released from the hydrolysis of triacylglycerol. In the adipose tissue in particular, the glycerol released from the hydrolysis of triacylglycerol cannot be re-utilized for the esterification of fatty acids because of absence of glycerol kinase. It has been proposed that during fasting adipose tissue generates the 3-glycerol phosphate required for triacylglycerol synthesis, either from glucose via glycolysis or, alternatively, from pyruvate via an abbreviated or truncated version of gluconeogenesis, termed glyceroneogenesis (4–7). The key enzyme in this pathway is the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (PEPCK;[1] EC 4.1.1.32). The transcription of the gene for PEPCK is stimulated by cAMP during periods of fasting (8, 9), resulting in an increase in enzyme activity in both adipose tissue and liver. In isolated epididymal adipose tissue from the rat, the rate of re-esterification of free fatty acids was greatly increased by the provision of a glyceroneogenic precursor such as pyruvate (10). In addition, hepatic glyceroneogenesis has been shown to account for ~89% of glyceride-glycerol in the triacylglycerol synthesized by rats fed a high protein diet (11).

There has not been a quantitative analysis of the relative rates of glyceride-glycerol synthesis from its precursors, plasma glycerol, pyruvate, or glucose in humans. In the present study we have quantified the relative contribution of plasma glycerol and pyruvate (plus lactate, alanine, etc.) to glyceride-glycerol in nonpregnant and pregnant women during fasting. Pregnant women were studied because of the higher concentration of plasma triacylglycerol during pregnancy, particularly in the third trimester. Our data show that the source of glyceride-glycerol following a brief fast is predominantly pyruvate. Because the synthesis of glucose and glyceride-glycerol

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[1] The abbreviations used are: PEPCK, the cytosolic form of phosphoenolpyruvate carboxykinase (GTP); FFA, free fatty acid; DHAP, dihydroxyacetone phosphate; HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrometry; VLDL, very low density lipoprotein.

from plasma glycerol share common enzymatic reactions, our data also suggest a functional separation of the pathways of glycerol entry into the liver and the 3-glycerol phosphate precursor pool for triacylglycerol synthesis.

EXPERIMENTAL PROCEDURES

Materials—[^{2}H]water, 99.9% ^{2}H, and [1,2,3-$^{13}C_3$]glycerol, 99% ^{13}C, were obtained from Isotech, Inc. (Miamisburg, OH).

Methods—The respective contributions of plasma glycerol and pyruvate were quantified in two separate groups of nonpregnant and pregnant women. Written informed consent was obtained from each woman and her spouse (when available) after fully explaining the procedure. The protocol was approved by the Institutional Review Board of the University Hospitals of Cleveland.

Glycerol Incorporation in Triacylglycerol—[1,2,3-^{13}C]Glycerol (over 99% ^{13}C) was infused in five normal nonpregnant women after an overnight fast. They were physically healthy and had a negative history of diabetes or other metabolic disorders in their family. The tracer glycerol was dissolved in normal saline, sterilized by Millipore filtration, and tested for pyrogenicity and sterility. All subjects reported to the Clinical Research Center at University of Hospitals of Cleveland following a 12-h fast. The tracer was infused at a constant rate of 0.03 mg/kg of body weight/min for a period of 5 h, following a priming dose of 0.5 mg/kg. Arterialized blood samples were obtained in heparinized syringes from the opposite arm at 30-min intervals starting at 1 h. Blood samples were centrifuged immediately, and the plasma samples were stored at −70 °C until analysis.

Pyruvate Incorporation into Triacylglycerol—The contribution of pyruvate to glycerol in triacylglycerol was evaluated using the total body water labeling method described for determining the rate of gluconeogenesis *in vivo* (12, 13). The volunteers had been studied previously, and the details of the experimental design and the data on glucose turnover and gluconeogenesis have been reported previously (12). Plasma samples for the quantification of glyceroneogenesis were examined in four nonpregnant women, five pregnant women in the first trimester (~11 weeks), and five women during the third trimester (34 weeks) of pregnancy. None of the women had any medical or obstetric illness or family history of diabetes and were not taking any medication. The subjects ate their last meals at 6:00 p.m. the evening before the day of the study. They were given [^{2}H]water orally (~3 gm/kg of body water), assuming total body water to be 55% of body weight (14), in three divided doses at 11:00 p.m., 3:00 a.m., and 7:00 a.m. Blood samples in heparinized syringes were obtained at frequent intervals starting at 8:00 a.m. and lasting until 12:00 noon. The samples were centrifuged immediately, and the separated plasma was stored at −70 °C until analysis.

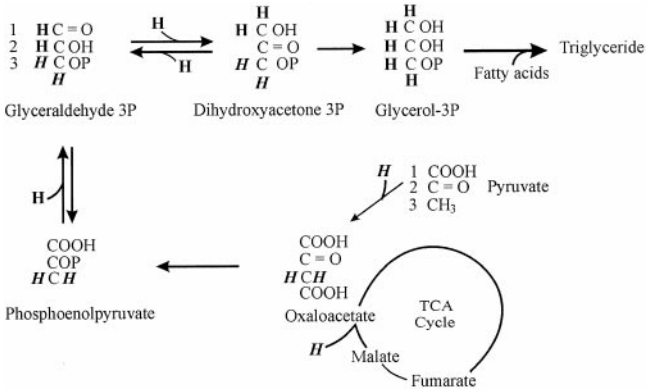

FIG. 1. **Conversion of pyruvate to glycerol-3-P and triacylglycerol and the site of labeling of the hydrogen atoms (*bold*) from deuterium in body water.** *TCA Cycle*, tricarboxylic acid cycle.

Analytical Methods—Total triacylglycerol with lipoproteins were precipitated with 10% perchloric acid and hydrolyzed with 0.5 N alcoholic potassium hydroxide at 70 °C. The samples were then neutralized, and glycerol, along with glucose, was isolated by ion exchange chromatography (3). Glycerol was then separated from glucose using HPLC by the following procedures. A Biorex Aminex column (HPX-87P), 300 × 7.8 mm, along with an Aminex-Q-1505 (action exchange resin in sodium form) guard column, was used. Glycerol was eluted isocretically using HPLC grade water at a flow rate of 0.2 ml/min at a column temperature of 85 °C. The glycerol fractions (from 52 to 60 min) were collected in a total volume of 1.6 to 1.8 ml.

[^{13}C] Enrichment of Glycerol—Glycerol from the plasma was isolated by ion exchange chromatography (3) whereas glycerol from triacylglycerol was isolated as described above. The isotopic enrichment of glycerol was measured by gas chromatography-mass spectrometry. A triacetate derivative of glycerol was prepared and analyzed using a Hewlett Packard GC-MS system (Model 5985A; Hewlett Packard Co., Palo Alto, CA) equipped with a capillary column (length, 10 m; inner diameter, 0.53 mm; film thickness, 1.2 μ; stationery phase, AT-1; Alltech Associates, Inc., Avondale, PA) (3). The GC conditions were as follows: injection temperature, 250 °C; initial oven temperature, 90 °C for 3 min; final temp, 230 °C; and ramp at 30 °C per min. Methane was used as a carrier and reactant gas. Chemical ionization was used with selected ion monitoring software. Peak areas for *m/z* 159, 160, 161, and 162, representing unlabeled and labeled isotopomer (m_0, m_{1-3}) of glycerol, were measured.

[^{13}C] Enrichment of Glucose—The m_3 and m_6 enrichment of glucose was measured by GC-MS; an aldonitrile penta-acetate derivative was prepared, and *m/z* 328 to 334, representing unlabeled and labeled isotopomers (m_0, m_{1-6}) of glucose, were quantified (15).

Measurement of Deuterium Enrichment in Glycerol—Carbons 1 and 3 of glycerol, along with their hydrogens, were cleaved by periodate oxidation to form formaldehyde, which was condensed with ammonium hydroxide to form hexamethylenetetramine (16). The latter was analyzed directly using a Hewlett Packard gas chromatography-mass spectrometry system (HP5970 equipped with an HP5890 gas chromatograph). The GC-MS conditions were as follows: a nonpolar polydimethyl siloxane stationary phase bonded fused-silica open-tubular column was used (AT-1; Alltech, Deerfield, IL). The column dimensions were length 30 m × 0.54-mm inner diameter, and film thickness was 1.2 μm. The injection temperature was 170 °C, initial temperature was 105 °C for 6 min, and final temperature was 230 °C; ramp rate was 45 °C per min. The retention time of hexamethylenetetramine was ~3.2 min. Electron impact ionization (70 eV) was used, and ions *m/z* 140 and 141 were monitored using the selected-ion monitoring technique. Standard solutions of hexamethylenetetramine prepared from [1-^{2}H]glucose of known enrichment were run along with unknowns to correct for instrumental variations (16). [^{2}H] enrichment of total body water was measured using the zinc reduction method with an isotope ratio mass spectrometer (12).

Calculations—The fractional contribution of glycerol to plasma glucose and triacylglycerol was calculated by comparing the m_3 enrichment of glucose and triacylglycerol with that of plasma glycerol. Because two trioses join together to form a glucose molecule, the enrichment in glucose was multiplied by 0.5.

The contribution of pyruvate to triacylglycerol was calculated from the deuterium enrichment of triacylglycerol using the following assumptions. The major sources of glycerol for triacylglycerol include

TABLE I
Contribution of plasma glycerol to glucose and triacylglycerol

[$^{13}C_3$]Glycerol was infused as prime constant rate infusion for a period of 5 h following an overnight fast, and the (m_3) enrichment was measured in glycerol, glucose, and triacylglycerol.

	Steady state (m_3) enrichment (%)			Fraction from glycerol (%)	
	Glycerol	Glucose	Triacylglycerol	Glucose	Triacylglycerol
Subject					
1	15.5	0.71	0.55	2.4	3.5
2	8.9	0.63	0.47	3.6	5.2
3	8.1	1.19	0.77	7.5	9.5
4	17.1	0.87	0.91	2.7	5.3
5	12.9	0.98	0.90	3.9	6.9
mean ± S.D.	12.5 ± 3.9	0.87 ± 0.22	0.72 ± 0.20	4.0 ± 2.1	6.1 ± 2.2

TABLE II

Glyceroneogenesis and gluconeogenesis from pyruvate in humans

The total body water was labeled by administering $[^2H]H_2O$ orally at ~3 gm/kg of estimated body water. Plasma samples for the measurements of deuterium enrichment in glucose C-6 and triacylglyceride glycerol were drawn between 4 and 6 h after the dose of labeled water. Subjects 1–4, nonpregnant women; 5–8, early pregnancy; 10–14, late pregnancy.

	Steady state deuterium enrichment			Glucose	Triacylglycerol
	Body water	Glucose C-6	Triacylglycerol	From pyruvate	From pyruvate
			%		
Subject					
1	0.491	0.467	0.73	47.56	45.6
2	0.276	0.263	0.32	47.64	10.5
3	0.291	0.248	0.39	42.61	28.9
4	0.279	0.203	0.33	36.38	12.9
5	0.461	0.44	0.64	47.72	35.6
6	0.325	0.373	0.54	57.38	61.5
7	0.313	0.37	0.39	69.11	19.8
8	0.317	0.318	0.45	50.16	37.2
9	0.425	0.46	0.58	54.12	32.9
10	0.453	0.34	0.34	37.53	
11	0.258	0.244	0.26	47.29	
12	0.307	0.355	0.34	57.82	5.9
13	0.265	0.330	0.33	62.26	18.9
14	0.26	0.316	0.31	60.7	13.5

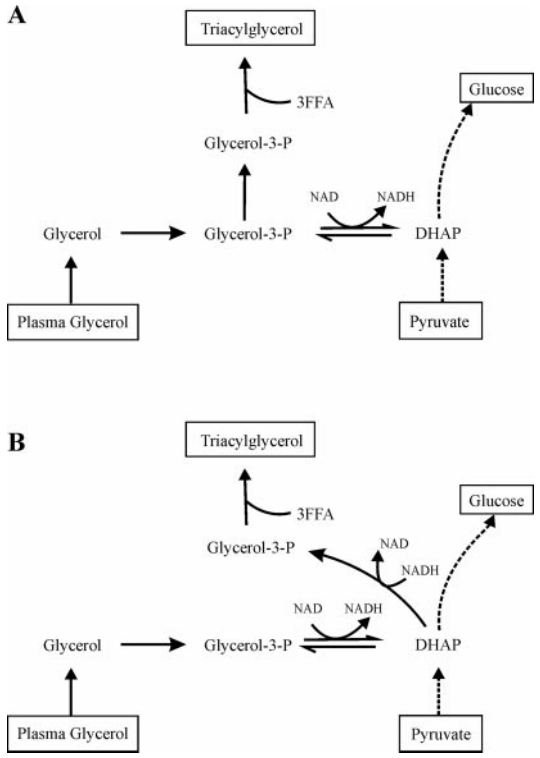

FIG. 2. **Pathway for the conversion of plasma glycerol to triacylglycerol or glucose in the liver.** *Panel A*, rapid flux from the triose pool. Glycerol enters the liver and is converted to glycerol-3-P by glycerol kinase, which is then oxidized to DHAP by glycerol-3-phosphate dehydrogenase. The labeled glycerol-3-P is in rapid equilibrium with DHAP. Because the flux of triosephosphate to glucose (~10 μmol/kg/min) exceeds that to triacylglycerol (<1 μmol/kg/min), more glycerol is ultimately converted to glucose than to triacylglycerol. *Panel B*, Two pools of glycerol-3-P. Glycerol enters the liver and is converted to glycerol-3-P, which is then oxidized to DHAP as outlined in *panel A*. A second pool of glycerol-3-P formed by the reduction of some of the DHAP is sequestered from the first pool and is utilized for the synthesis of hepatic triacylglycerol (see "Results and Discussion").

pyruvate (plus lactate, alanine, and other gluconeogenic amino acids) and plasma glucose or glycerol. The methyl hydrogens (C3) of pyruvate that form C6 of glucose and C3 of glyceraldehyde-3-P exchange with hydrogens in body water so that $[^2H]$ enrichment of hydrogens bound to C3 of pyruvate or to phosphoenolpyruvate is similar to that of water (Fig. 1). This assumption was evaluated in fasting human subjects and shown to be over 80% complete (17). One hydrogen on C1 of dihydroxyacetone phosphate (DHAP), the immediate precursor of glycerol-3-P, is obtained from body water during the conversion of phosphoenolpyruvate to glyceraldehyde-3-P. The second hydrogen (on C1) is also obtained from body water during the isomerization of DHAP and glyceraldehyde-3-P so that the $[^2H]$ enrichment of both the hydrogens on C1 of glycerol-3-P is the same as that of body water. Thus the $[^2H]$ enrichments of hydrogen on C1 and C3 of triacylglycerol formed from pyruvate will be the same as that of the body water. In contrast, the triacylglycerol formed from nonpyruvate sources will have the hydrogens labeled only at the C1 position (Fig. 1). Periodate oxidation results in cleavage of both C1 and C3 of glycerol, along with the attached hydrogen. Therefore the measured ^2H enrichment in glycerol is the sum of the enrichments of hydrogen from pyruvate and nonpyruvate sources. From the assumptions presented above, *i.e.* the deuterium enrichment of hydrogen on glycerol carbons from pyruvate will be the same as water and that the deuterium enrichment of hydrogen on nonpyruvate carbon source will be that of water (C1), and the natural abundance of deuterium (C3), the relative flux of pyruvate and nonpyruvate carbon to triacylglycerol can be calculated.

RESULTS AND DISCUSSION

Contribution of Plasma Glycerol—The mean m_3 enrichments of plasma glycerol, glucose, and triacylglycerol during 3.5 to 5 h of $[^{13}C_3]$glycerol infusion are presented in Table I. An isotopic steady state was evident in all three metabolic pools. As shown, only 6.1% of the triacylglycerol pool was derived from plasma glycerol in normal healthy women after 17 h of fasting; the rest was from nonglycerol sources. The rate of appearance of glycerol, calculated from tracer dilution, was 1.65 ± 0.6 μmol/kg/min (mean ± S.D.). Approximately 4% of the plasma glucose pool was derived from plasma glycerol. Assuming the rate of glucose turnover to be ~10 μmol/kg of body weight/min (12), this would represent ~0.8 μmol of glycerol/kg/min or 50% of glycerol turnover. In contrast, if the rate of appearance of VLDL triacylglycerol is assumed to be ~0.5 μmol/kg/min (18), the contribution of glycerol to triacylglycerol synthesis would represent 0.03 μmol/kg/min or less than 2% of the glycerol turnover.

Contribution of Pyruvate to Triacylglycerol—The steady state deuterium enrichment in body water in triacylglycerol and carbon-6 of glucose are displayed in Table II. The ^2H enrichment in triacylglycerol was measured ~5 h after the last dose of $[^2H]$water and does not represent an isotopic plateau in the triacylglycerol pool. The fractional contribution of pyruvate to triacylglycerol ranged from 10 to 46% in nonpregnant women and from 20 to 60% during early gestation. In late gestation, 6 to 19% of triacylglycerol was derived from pyruvate. It was not measurable in two subjects. The ^2H enrichment of triacylglycerol was lower in women studied late in gestation, most likely

a consequence of the large plasma triacylglycerol pool at this stage in pregnancy (19). As shown in Table II, 36 to 69% of glucose was produced via gluconeogenesis from pyruvate. No statistically significant differences were evident in any of the parameters between pregnant and nonpregnant subjects.

The data from the present study show that in nonpregnant women, 6.1% of glycerol in triacylglycerol was derived from plasma glycerol and that a significant portion (10 to 60%) of the triacylglycerol was derived from pyruvate (glyceroneogenesis). The large variance observed was most likely because of a lack of isotopic steady state in the triacylglycerol pool during [^{2}H]water studies, because the plasma samples were obtained after a relatively short period following tracer administration, and because of the slow turnover rate of the triacylglycerol pool.

Data from the present study are comparable with the recently published work by Botion *et al.* (11), in which the rates of glyceroneogenesis were quantified in rats *in vivo*. Hepatic glyceroneogenesis accounted for 80% of the total glyceride-glycerol formation in rats fed a high protein diet. However, they measured total [^{3}H] incorporation into all hydrogens of glycerol following [^{3}H]water administration to rats and likely overestimated the contribution of glyceroneogenesis.

The physiological role of glyceroneogenesis from pyruvate in the regulation of hepatic triacylglycerol synthesis remains to be determined. Knopp *et al.* (20) examined the effect of FFA availability and of insulin on VLDL triglyceride production and VLDL apoB production in healthy young men. Using the euglycemic hyperinsulinemic clamp method they showed that raising the plasma FFA levels by infusion of intralipid could not completely counter the inhibitory effect of insulin on VLDL triglyceride and apoB production. In contrast, elevation of FFA alone, without insulin, acutely stimulated VLDL production in healthy young males. The authors concluded that the acute inhibition of VLDL production by insulin *in vivo* is only partly because of the suppression of plasma FFA release by adipose tissue and may also be because of an FFA-independent process. Other investigators (21) have shown a defective regulation of triacylglycerol metabolism by insulin in noninsulin-dependent diabetic subjects and concluded that the inability of insulin to acutely inhibit the release of VLDL triglycerides from liver, despite efficient suppression of serum FFA, contributes to the hypertriglyceridemia in noninsulin-dependent diabetic subjects. We speculate that the acute suppression of VLDL triacylglycerol synthesis by insulin is an integrated response as a result of insulin action at multiple sites; these include the peripheral suppression of lipolysis and suppression of PEPCK gene expression resulting in a reduction in glyceroneogenesis, along with a reduction in gluconeogenesis. The continued high triacylglycerol synthesis observed in noninsulin-dependent diabetic subjects during hyperinsulinemia may be related to hepatic insulin resistance and lack of suppression of PEPCK gene transcription. These findings, together with the data in the present paper, suggest that glyceroneogenesis may play an important role in controlling VLDL production by the liver.

During periods of starvation, glycerol is released by lipolysis in adipose tissue. Because of the absence of glycerol kinase, this glycerol cannot be further metabolized by adipose tissue and is released into circulation and is taken up by the liver for the synthesis of glucose. Because the liver also synthesizes a significant amount of triacylglycerol during fasting, it would be logical to assume that this glycerol would be the major source of the glyceride-glycerol for triacylglycerol synthesis. Our data show that only a small fraction of the glyceride-glycerol made by the liver during fasting is synthesized from glycerol, this despite the fact that a large fraction of the glycerol (50%) is converted to glucose.

We can think of two explanations for our findings that only a small fraction of the plasma glycerol entering the liver is converted to glyceride-glycerol, despite the fact that it enters a potential precursor pool of 3-glycerol phosphate (Fig. 2). First, a single pool of 3-glycerol phosphate is in very rapid equilibrium with the triosephosphate pool, so that there is a rapid labeling of the triosephosphate pool by the infused glycerol tracer. Because the flux from triosephosphate to glucose during fasting ($\sim$10 μmol/kg/min) far exceeds that to triacylglycerol ($<$1 μmol/kg/min), more glycerol will end up in glucose than in triacylglycerol (Fig. 2*A*). For the same reason, because the contribution of pyruvate to the triosephosphate pool is much greater than that of plasma glycerol, the contribution of pyruvate to triacylglycerol will be far greater than that of glycerol, as was observed in the present study. Second, there may be two functional pools of 3-glycerol phosphate in the liver. One of these pools is the precursor for triosephosphate, and the other is the precursor pool for glyceride-glycerol synthesis (Fig. 2*B*). The first suggested scenario seems more likely, because it is supported by the observation of Siler *et al.* (22) that ethanol consumption by human subjects resulted in an accumulation of [2-$^{13}C_1$]glycerol from plasma in hepatic glycerol-3-P. This result was due in part to the effect of the ethanol-induced increase in the cytosolic NADH level on the equilibrium position of glycerol-3-phosphate dehydrogenase. As predicted, this alteration in the cytosolic redox state markedly increased the labeled glycerol in glyceride-glycerol, because it effectively reduces DHAP to glycerol-3-P, which in turn serves as a precursor for triacylglycerol synthesis. Clearly more research is required to resolve the pathways of carbon flow responsible for the low level of conversion of plasma glycerol to glyceride-glycerol in the livers of fasting humans.

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