Role of glycerol 3-phosphate and glycerophosphate acyltransferase in the nutritional control of hepatic triacylglycerol synthesis

Peter E. DECLERCQ, Luc J. DEBEER and Guy P. MANNAERTS

Afdeling Farmakologie, Fakulteit Geneeskunde, Katholieke Universiteit Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

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1. Glycerol 3-phosphate content of isolated hepatocytes from starved rats and of glycogen-depleted hepatocytes from fed rats was low and severely limited triacylglycerol synthesis. 2. Raising the glycerol 3-phosphate content by addition of precursors to the cells resulted in a hyperbolic-like relationship between triacylglycerol synthesis and cellular glycerol 3-phosphate content. Statistical analysis of the curves showed no significant differences between the nutritional states either at saturating or at subsaturating glycerol 3-phosphate content. 3. $V_{\max}$ of glycerophosphate acyltransferase measured in homogenized hepatocytes was decreased by 30–40% in starvation. There was no change in apparent K_m for glycerol 3-phosphate. Since at saturating glycerol 3-phosphate content esterification rates in hepatocytes of both nutritional states were identical, the enzyme is not limiting esterification under this condition. 4. At subsaturating glycerol 3-phosphate content the flux through glycerophosphate acyltransferase necessarily limits esterification. Therefore one would expect a decrease in esterification in starvation under this condition. This was the case when triacylglycerol synthesis was plotted against intracellular glycerol 3-phosphate concentration, calculated from the cellular glycerol 3-phosphate content and the intracellular water space, which was smaller in hepatocytes from starved rats. 5. The data obtained in hepatocytes were extrapolated to the intact liver by using the number of parenchymal cells per g of liver as determined from marker-enzyme analysis and the liver weight per 100 g body weight. The extrapolation suggested that glycerol 3-phosphate is limiting esterification *in vivo* for contents below 0.3–0.4 and 0.5–0.65 $\mu\text{mol/g}$ for livers from fed and starved animals respectively. Also for a given fatty acid load and a glycerol 3-phosphate content below 0.3 $\mu\text{mol/g}$ the liver may esterify less in the starved state. However, at the glycerol 3-phosphate contents measured in freeze-clamped livers (0.30 and 0.44 $\mu\text{mol/g}$ for the fed and starved state respectively), livers in both nutritional states seemed capable of esterifying similar amounts of fatty acids.

Plasma long-chain non-esterified fatty acids are metabolized by the liver via two major pathways: (1) oxidation mainly to ketones and CO_2 , and (2) esterification with glycerol 3-phosphate to form triacylglycerols and phospholipids. The partitioning of the incoming fatty acids between oxidation and esterification is under nutritional and hormonal control. Perfused livers or isolated hepatocytes from fed rats esterify more fatty acids than they oxidize, whereas the converse is true for liver preparations from starved rats (Mayes & Felts, 1967; McGarry & Foster, 1971; Ontko, 1972). Theoretically, the balance between oxidation and esterification can be altered by changes in the activities of either the

oxidation or the esterification pathway, or by simultaneous reciprocal changes in the activity of both pathways. The activities of the oxidation pathway appears to be controlled by malonyl-CoA, a potent inhibitor of carnitine palmitoyltransferase I. In the fed state, in which lipogenesis is high, the increased intracellular concentration of malonyl-CoA suppresses fatty acid oxidation and thereby prevents the newly synthesized fatty acids from being oxidized. In the starved animal lipogenesis and malonyl-CoA decrease, so that the inhibition of fatty acid oxidation is relieved (McGarry & Foster, 1980). It is not clear whether the control exerted on oxidation suffices to explain the reciprocal changes

in fatty acid oxidation and esterification, or whether an as yet unknown additional control on esterification has to be postulated.

In the starved state there is a decrease in the activity of glycerophosphate acyltransferase, the first enzyme committed to esterification (Aas & Daas, 1971; Mangiapane *et al.*, 1973; Van Tol, 1974). The availability of glycerol 3-phosphate has been proposed as another regulatory factor in fatty acid esterification (Fritz, 1961; Wieland & Matschinsky, 1962; Tzur *et al.*, 1964; Mayes & Felts, 1967; Exton & Park, 1967; Van Tol, 1974; Christiansen, 1979; Beynen *et al.*, 1980; Lund *et al.*, 1980; Sugden *et al.*, 1980; Williamson *et al.*, 1980; Wirthensohn *et al.*, 1980). However, in none of these experiments was it established that glycerol 3-phosphate was in fact limiting esterification. More recently we determined the content below which glycerol 3-phosphate limits esterification in hepatocytes from starved rats (Debeer *et al.*, 1981). In the present experiments we measured the concentration range within which it limits esterification in hepatocytes from fed rats and compared it with similar data obtained in hepatocytes from starved rats. In addition, we explored the possible regulatory role of the nutritional changes in glycerophosphate acyltransferase activity.

Experimental

Animals

Male Wistar rats weighing 150–200 g and maintained on a standard laboratory diet were used. Starved rats were deprived of food for 24 h. All experiments were initiated between 08:00 and 09:00 h.

Triacylglycerol synthesis in isolated hepatocytes

Hepatocytes were isolated as described previously (Thomas *et al.*, 1978). To lower the glycogen and glycerol 3-phosphate contents of freshly isolated hepatocytes from fed rats, the cells (10^7 /ml) were suspended in Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, containing 2.6% defatted albumin and preincubated without shaking for 90 min at 37°C in closed flasks with O_2/CO_2 (19:1) as the gas phase. At 30, 60 and 90 min the cells were washed by centrifugation (75 g, 1 min) and resuspended in the same albumin-containing buffer. Cells from starved rats were treated similarly. The washed cells were adjusted to 2.5×10^6 cells/ml with the same buffer and incubated with shaking in the presence of 1 mM-[1- ^{14}C]palmitate (sp. radioactivity 1 Ci/mol) and various glycerol 3-phosphate precursors as indicated in the Figures. Incubations were stopped after 10 or 20 min, either with $HClO_4$ for the determination of glycerol 3-phosphate (Debeer *et al.*, 1981) and radioactive acid-soluble oxidation pro-

ducts (Mannaerts *et al.*, 1979), or with chloroform/methanol (2:1, v/v) for the measurement of incorporation of radioactivity in triacylglycerol (Mannaerts *et al.*, 1979). Under the conditions employed, glycerol 3-phosphate content reached similar values at 10 and 20 min, indicating that esterification was operating under steady-state conditions. Therefore glycerol 3-phosphate values given in the Figures are means of the values measured at both time points. Triacylglycerol synthesis is expressed as rates of palmitate incorporation between the same two time points.

Determination of the intracellular water space of isolated hepatocytes

This was determined as the $[U-^{14}C]$ -3-*O*-methylglucose-accessible space (Crack & Elliott, 1979). Isolated hepatocytes suspended in albumin-containing Krebs–Henseleit buffer (10^7 /ml) were incubated with 2.5 μ Ci of $[U-^{14}C]$ -3-*O*-methylglucose (sp. radioactivity 310 Ci/mol) for 3 min at 37°C and then centrifuged through a layer of bromododecane and dodecane as described by Cornell (1980). The cell pellet was solubilized in Soluene 350 and counted for radioactivity by liquid scintillation spectrometry. Adhering extracellular water was measured with $[G-^3H]$ inulin (sp. radioactivity 0.14 Ci/g) and subtracted. The intracellular water spaces of freshly isolated hepatocytes and of hepatocytes that had been preincubated for 90 min were identical.

Determination of the number of parenchymal cells per g of intact liver

This was calculated from determinations in whole liver homogenates and in homogenates from isolated hepatocytes of the activities of glucose 6-phosphatase, succinate dehydrogenase and glutamate dehydrogenase. These enzymes are largely confined to parenchymal cells (Van Berkel & Koster, 1977) and can therefore be considered as markers for these cells. Since the calculations based on each separate marker agreed within 10%, the values given in the text are means based on all three markers.

Preparation of homogenates and cell-fractionation studies

Isolated hepatocytes (10^7 /ml) suspended in 0.25 M-sucrose containing 1 mM-dithiothreitol, 1 mM-EDTA and 10 mM-Tris/HCl, pH 7.4, were disrupted by sonication for 2 min (Branson Sonifier Cell Disrupter B 15, output 4, 20% pulse time). Whole liver homogenates (25%, w/v) were prepared in the same mixture in a Potter–Elvehjem homogenizer with two strokes of a motor-driven pestle (1200 rev/min) and fractionated into a nuclear, total mitochondrial and microsomal fraction and soluble components as described by de Duve *et al.* (1955).

Enzyme measurements

Glutamate dehydrogenase (EC 1.4.1.3, marker enzyme for mitochondrial matrix), carnitine palmitoyltransferase (EC 2.3.1.21, mitochondrial inner membrane) and glucose 6-phosphatase (EC 3.1.3.9, endoplasmic reticulum) were determined as described previously (Mannaerts *et al.*, 1979; Debeer *et al.*, 1979). Monoamine oxidase (EC 1.4.3.4, mitochondrial outer membrane), NADPH-dependent cytochrome *c* reductase (EC 1.6.2.4, endoplasmic reticulum) and succinate dehydrogenase (EC 1.3.99.1, mitochondrial inner membrane) were assayed by the methods of Weissbach *et al.* (1960), Beaufay *et al.* (1974) and Sottocasa *et al.* (1967) respectively.

Glycerophosphate acyltransferase (acyl-CoA: *sn*-glycerol 3-phosphate *O*-acyltransferase, EC 2.3.1.15) was measured by the method of Bates & Saggerson (1977), with the following modifications. The assay mixture, in a final volume of 1 ml, contained KCl (120 mM), Tris/HCl, pH 7.4 (50 mM), palmitoyl-CoA (65 μ M), defatted bovine serum albumin 6 or 1.2 mg (molar ratios of palmitoyl-CoA/albumin of 0.65 and 3.25 respectively), L-[U-¹⁴C]glycerol 3-phosphate (sp. radioactivity 0.5 Ci/mol; concentration range 0.1–5 mM), dithiothreitol (0.7 mM) and appropriate quantities of enzyme source. The enzyme was preincubated for 6 min at 30°C in the reaction mixture described above, except that palmitoyl-CoA was absent. Reactions were started with the addition of palmitoyl-CoA and the incubations were continued for another 6 min at 30°C. Reactions were terminated with water-saturated butanol and the butanol-soluble radioactivity was determined. Appearance of butanol-soluble radioactivity was linear with time for the incubation period used. $V_{\max}$ and apparent K_m values for glycerol 3-phosphate were calculated by using the Woolf–Hofstee linear transformation of the hyperbolic relationship obtained between enzyme activity and glycerol 3-phosphate concentration (Hofstee, 1959).

Protein was determined by a modification (Peterson, 1977) of the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of glycerol 3-phosphate content of liver *in vivo*

When glycerol 3-phosphate contents of livers *in vivo* were measured, rats were anaesthetized with Nembutal (50 mg/kg intraperitoneally) and their livers excised and clamped between metal tongs precooled in liquid nitrogen (Wollenberger *et al.*, 1960). The time period between excision and freeze-clamping never exceeded 5 s. Longer time periods caused erratically high results. Glycerol 3-phosphate was measured spectrophotometrically on the neutralized HClO₄ extracts (Hohorst, 1970).

Determination of plasma non-esterified fatty acids and triacylglycerol entry into the circulation

Plasma non-esterified fatty acids were measured by the method of Duncombe (1963). Triacylglycerol entry into the circulation was estimated *in vivo* by the Triton WR-1339 method as described by Otway & Robinson (1967). Plasma was extracted with chloroform/methanol (2:1, v/v) and analysed for triacylglycerols as described previously (Debeer *et al.*, 1977).

Materials

Radioactive products were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V, powder), collagenase (type I, from *Clostridium histolyticum*) and cytochrome *c* (type III, horse heart) were from Sigma Chemical Co., St. Louis, MO, U.S.A. The fraction V albumin was defatted by the method of Chen (1967), dialysed in the cold for 48 h against water and freeze-dried. Triton WR-1339 was from Serva, Heidelberg, Germany; Insta-Gel II and Soluene 350 were from Packard Instrument Co., Downers Grove, IL, U.S.A. Carnitine, dithiothreitol and palmitoyl-CoA were purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Enzymes and coenzymes used in the various determinations were obtained from Boehringer G.m.b.H., Mannheim, Germany. All other reagents were of analytical grade.

Statistical analysis

The curves of Fig. 1 were drawn after statistical analysis of the experimental data by means of computer programs BMDP 1R and BMDP 3R for regression analysis of the Biomedical Library Statistical Software, Department of Biostatistics, University of California, Los Angeles, CA, U.S.A. It was assumed that the rates of triacylglycerol synthesis versus glycerol 3-phosphate content of isolated hepatocytes followed hyperbolic kinetics. After linear transformation by the Woolf–Hofstee method (Hofstee, 1959), a linear regression analysis of the data was performed to find the most likely values of (1) the apparent K_m for glycerol 3-phosphate, (2) the apparent $V_{\max}$ of triacylglycerol synthesis and (3) the intercept of the curve with the abscissa (see Fig. 1). These three parameters were then used as starting values for a non-linear regression analysis which allowed the calculation of the most likely rate of triacylglycerol synthesis for each corresponding experimentally determined glycerol 3-phosphate value. The confidence limits of the curves were calculated as follows:

$$\text{confidence limits} = Y \pm (t_{n-3} \cdot \text{S.D.}),$$

where Y is the computed value of the rate of triacylglycerol synthesis, t_{n-3} is the t value at the

0.05 level of significance for $n-3$ degrees of freedom, n is the number of experimental data and S.D. is the standard deviation of Y .

Results and discussion

Glycerol 3-phosphate dependence of triacylglycerol synthesis in isolated hepatocytes from fed and starved rats

We previously demonstrated that glycerol 3-phosphate availability seriously limits fatty acid esterification in isolated hepatocytes from starved rats incubated in the absence of carbohydrate (Debeer *et al.*, 1981). The glycerol 3-phosphate content of such cells is approx. $0.15 \mu\text{mol}/10^8$ cells. Addition of precursors such as lactate, glucose or glycerol increased glycerol 3-phosphate content together with the rates of triacylglycerol synthesis, and revealed a hyperbolic-like relationship between both parameters. That the increased rates of triacylglycerol synthesis after addition of precursors were most probably due to the increase in glycerol 3-phosphate and not to a possible allosteric activation of one of the enzymes of the esterification pathway by a glycolytic-gluconeogenic intermediate that might rise concomitantly was shown by the fact that the incorporation of radioactive palmitate was enhanced into all intermediates (lysophosphatidate, phosphatidate and diacylglycerol). Below $0.4 \mu\text{mol}$ of glycerol 3-phosphate/ 10^8 cells, rates of triacylglycerol synthesis steeply increased with increasing glycerol 3-phosphate and then started to reach a plateau. Freshly isolated hepatocytes from fed rats contained approx. $0.4 \mu\text{mol}$ of glycerol 3-phosphate/ 10^8 cells, which was sufficient to allow

near-maximal rates of esterification (Debeer *et al.*, 1981). Since we wanted to compare the glycerol 3-phosphate dependence of triacylglycerol synthesis in hepatocytes from fed and starved animals, the hepatocytes from fed rats were first preincubated for 90 min to deplete their glycogen and lower their glycerol 3-phosphate content. Hepatocytes from starved rats were treated similarly. The hepatocytes were then incubated in the presence of 1 mM-palmitate and of lactate plus pyruvate or glucose to raise their glycerol 3-phosphate content, and rates of triacylglycerol synthesis were measured. A high palmitate concentration was used, since at elevated palmitate concentrations oxidation rates become equally high in hepatocytes from fed and starved rats. In the present experiments 0.42 ± 0.02 and $0.41 \pm 0.02 \mu\text{mol}$ of palmitate were oxidized/min per 10^8 cells for hepatocytes from fed and starved rats respectively, so that differences in oxidation did not influence triacylglycerol synthesis indirectly by altering the intracellular acyl-CoA concentration. The high oxidation rates in hepatocytes from fed rats are explained by the inhibition of acetyl-CoA carboxylase and malonyl-CoA synthesis by the increased intracellular acyl-CoA (McGarry & Foster, 1980). Fig. 1 shows the relationship between cellular glycerol 3-phosphate and rates of triacylglycerol synthesis in isolated hepatocytes from fed and starved animals. The data reveal that below $0.4-0.5 \mu\text{mol}$ of glycerol 3-phosphate/ 10^8 cells triacylglycerol synthesis sharply increased with increasing glycerol 3-phosphate content in both the fed and the starved state. The curves, which were computer-drawn after non-linear-regression analysis of the data, are again represented in Fig. 2 together

Fig. 1. Esterification rate versus intracellular glycerol 3-phosphate content of isolated hepatocytes from fed and starved rats

Isolated hepatocytes were preincubated as described in the Experimental section to lower their glycerol 3-phosphate content. The cells were then incubated in the presence of $[1-^{14}\text{C}]$ palmitate and with the following glycerol 3-phosphate precursors: none ($\circ, \bullet$); lactate (5 mM) plus pyruvate (0.5 mM) ($\triangle, \blacktriangle$); glucose (20 mM) ($\nabla, \blacktriangledown$). The curves are the result of a non-linear-regression analysis of the individual data (correlation coefficient, r : 0.82, fed; 0.88, starved) obtained from six (starved) or seven (fed) cell preparations.

with their 95% confidence limits. Fig. 2 shows a wide overlap in confidence intervals, indicating that both curves were not significantly different. Although this may be due to the relatively wide scatter, inherent to such experiments, of the experimental points, the data nevertheless demonstrate that the nutritional state of the animal causes no large difference in glycerol 3-phosphate dependence of triacylglycerol synthesis. This may seem unexpected, however, since several authors have described a decreased activity of glycerophosphate acyltransferase in the starved state (Aas & Daae, 1971; Mangiapane *et al.*, 1973; Van Tol, 1974). Evidently, at limiting glycerol 3-phosphate content the flux through this enzymic step limits esterification and one would therefore expect to observe a slower increase of triacylglycerol synthesis with increasing cellular glycerol 3-phosphate content in cells from starved animals.

Glycerophosphate acyltransferase activity in homogenates of isolated hepatocytes from fed and starved rats

Glycerophosphate acyltransferase activity is found at the mitochondrial outer membrane and in endoplasmic reticulum (Daae & Bremer, 1970; Monroy *et al.*, 1972; Nimmo, 1979a,b). It has been reported that the microsomal enzyme is strongly inhibited by thiol-blocking reagents such as *N*-ethylmaleimide, whereas the mitochondrial enzyme appears to be insensitive to these agents (Monroy *et al.*, 1972; Bates *et al.*, 1977; Nimmo, 1979a). In a first series of experiments we confirmed these findings. After centrifugal fractionation of homogenates, glycerophosphate acyltransferase activity was measured in the presence and absence of 2 mM-*N*-ethylmaleimide in the subcellular fractions. *N*-Ethylmaleimide-insensitive activity followed the subcellular distribution of the mitochondrial markers glutamate dehydrogenase, carnitine palmitoyl-transferase and especially the outer-membrane marker monoamine oxidase (results not shown), confirming its mitochondrial nature. The distribution of *N*-ethylmaleimide-sensitive activity closely paralleled the distribution of the microsomal markers glucose 6-phosphatase and cytochrome *c* reductase (results not shown). Therefore all further measurements of glycerophosphate acyltransferase activity were performed in whole cell homogenates in the presence and absence of *N*-ethylmaleimide, and *N*-ethylmaleimide-insensitive and -sensitive activities were taken as the mitochondrial and microsomal activities respectively. Fig. 3 shows glycerophosphate acyltransferase activities of isolated hepatocytes from fed (panels *a* and *c*) and starved (panels *b* and *d*) rats measured at a palmitoyl-CoA/albumin molar ratio of 0.65 (panels *a* and *b*) and 3.25 (panels *c* and *d*). In hepatocytes from starved rats there was a significant ($P < 0.05$) decrease of 30–40% in total

Fig. 2. Confidence limits of esterification rate versus intracellular glycerol 3-phosphate content of isolated hepatocytes from fed (●) and starved (○) rats

The solid curves are the curves of Fig. 1. The broken curves represent the corresponding 95% confidence limits calculated as described in the Experimental section

and mitochondrial glycerophosphate acyltransferase activities at all glycerol 3-phosphate concentrations. Although the microsomal activity displayed a similar decrease, it reached statistical significance at only two glycerol 3-phosphate concentrations. This lack of statistical significance may be the result of a larger variance owing to the indirect estimation method. The decrease in total and mitochondrial activities in the starved state is in agreement with the work of others (Aas & Daae, 1971; Van Tol, 1974; Zammit, 1981). Mangiapane *et al.* (1973) also observed a decrease in microsomal glycerophosphate acyltransferase activity in starvation when activity was expressed per unit of DNA. Expressed in terms of protein, no decrease in microsomal activity was found by Fallon & Kemp (1968) and by Zammit (1981). However, owing to a decrease in the liver's total protein content during starvation, the liver's total microsomal activity may also have been lower in those studies. Table 1 gives apparent K_m values for glycerol 3-phosphate and maximal

Fig. 3. *Glycerophosphate acyltransferase activity in homogenates of isolated hepatocytes from fed and starved rats* Glycerophosphate acyltransferase was determined in homogenized hepatocytes from fed (a and c) and starved rats (b and d) and expressed as μmol of radioactive glycerol 3-phosphate incorporated into butanol-extractable products. The palmitoyl-CoA/albumin molar ratio was 0.65 (a and b) or 3.25 (c and d). ●, Total activity; ■, mitochondrial activity; ▲, microsomal activity. The plotted values are means of at least five determinations. S.E.M. values, which are not shown, were generally between 5 and 15% of the corresponding values. The total and mitochondrial activities were significantly ($P < 0.05$) decreased at all glycerol 3-phosphate concentrations in hepatocytes from starved rats compared with those from fed rats. A statistically significant decrease in microsomal activity was only observed at glycerol 3-phosphate concentrations of 0.5 mM (palmitoyl-CoA/albumin ratio 0.65) and 0.2 mM (palmitoyl-CoA/albumin ratio 3.25).

Table 1. *Kinetic parameters of glycerophosphate acyltransferase in homogenates from isolated hepatocytes* Glycerophosphate acyltransferase was assayed in homogenates of isolated hepatocytes from fed and starved animals as described in the Experimental section. 'Total' and 'mitochondrial' refer to the activities of the enzyme in the absence and presence of *N*-ethylmaleimide respectively. 'Microsomal' refers to the difference between total and mitochondrial activities. Apparent K_m and $V_{\max}$ were calculated from the linear transformation of the curves represented in Fig. 3.

Palmitoyl-CoA/ albumin molar ratio	Glycerophosphate acyltransferase activity	Apparent K_m (mM)		$V_{\max}$ (nmol of glycerol 3-phosphate incorporated/min per 10^8 cells)	
		Fed	Starved	Fed	Starved
0.65	Total	0.15	0.16	361	244
	Mitochondrial	0.17	0.11	209	115
	Microsomal	0.18	0.17	178	120
3.25	Total	0.41	0.48	658	487
	Mitochondrial	0.24	0.36	222	162
	Microsomal	0.52	0.54	434	328

velocities calculated from the average curves represented in Fig. 3. The apparent K_m values were very similar in hepatocytes from fed and starved animals; an approx. 3-fold increase was observed when the

palmitoyl-CoA/albumin ratio was increased from 0.65 to 3.25. Since it has been reported that the rat adipose-tissue glycerophosphate acyltransferase can be inactivated in a phosphorylation reaction

catalysed by cyclic AMP-dependent protein kinase (Nimmo & Houston, 1978), glycerophosphate acyltransferase was also measured in homogenates prepared in the presence of 50 mM-NaF to inhibit possible phosphoprotein phosphatase activity. The presence of NaF did not affect the kinetic parameters.

Triacylglycerol synthesis versus intracellular glycerol 3-phosphate concentration in hepatocytes from fed and starved animals

Comparison of Figs. 2 and 3 reveals two apparent discrepancies. (1) Although the maximal activity of glycerophosphate acyltransferase is decreased in starvation, the rate of triacylglycerol synthesis at saturating glycerol 3-phosphate content is not. This indicates that under the latter condition another enzyme of the esterification sequence limits triacylglycerol synthesis. Our observations therefore indirectly support the idea that phosphatidate phosphohydrolase may determine the rate of triacylglycerol synthesis when substrate supply is high (for review, see Brindley, 1978). (2) Evidently, at limiting glycerol 3-phosphate content the flux through the glycerophosphate acyltransferase step is limiting esterification, and under this condition one would expect lower rates of triacylglycerol synthesis in hepatocytes from starved animals. To solve this discrepancy we determined the water space of isolated hepatocytes so that glycerol 3-phosphate content of the cells could be converted into intracellular concentrations. The intracellular water space, determined as the [^{14}C]methylglucose-accessible space, was $419.7 \pm 23.1 \mu\text{l}/10^8$ cells ($n = 22$) and $301.5 \pm 16.6 \mu\text{l}/10^8$ cells ($n = 23$) for hepatocytes from fed and starved animals respectively ($P < 0.001$). Thus, because of their smaller water space, hepatocytes from starved rats have higher intracellular glycerol 3-phosphate concentrations at a given glycerol 3-phosphate content than do hepatocytes from fed rats. In Fig. 4 the rates of triacylglycerol synthesis taken from Fig. 2 are plotted against intracellular glycerol 3-phosphate concentrations calculated from the measured glycerol 3-phosphate content and the intracellular water space. The curve for hepatocytes from starved rats is now shifted to the right relative to the curve for hepatocytes from fed rats. Between 0.5 mM and 1 mM-glycerol 3-phosphate the 95% confidence intervals do not overlap, indicating that both curves are significantly different. Thus for a given intracellular glycerol 3-phosphate concentration below 1 mM, hepatocytes from starved rats esterify less than do hepatocytes from fed rats. Since the apparent K_m for glycerol 3-phosphate of glycerophosphate acyltransferase does not change on starvation, the decreased esterification appears to be the result of the decrease in maximal velocity of the

Fig. 4. Esterification rate versus intracellular glycerol 3-phosphate concentration in isolated hepatocytes from fed (●) and starved (○) rats

Rates of triacylglycerol synthesis in Fig. 2 were plotted against intracellular glycerol 3-phosphate concentrations calculated from the corresponding glycerol 3-phosphate content and the intracellular water space. The broken curves represent the 95% confidence limits calculated as described in the Experimental section.

enzyme. Interestingly, below 1 mM-glycerol 3-phosphate there is a 30–40% decrease in triacylglycerol synthesis in hepatocytes from starved as compared with fed rats, a percentage very similar to the decrease in glycerophosphate acyltransferase activity. Since at higher glycerol 3-phosphate concentrations another enzyme of the pathway seems to become rate-limiting, the decrease in maximal velocity of glycerophosphate acyltransferase does not appear as a decrease in maximal activity but as an increase in apparent K_m for glycerol 3-phosphate of the overall pathway of triacylglycerol synthesis (see Fig. 4).

Extrapolation to the situation in vivo

The data obtained in isolated hepatocytes (Fig. 2) were extrapolated in an attempt to describe the glycerol 3-phosphate dependence of fatty acid esterification *in vivo*. This is shown in Fig. 5. Rates of triacylglycerol synthesis were expressed per 100 g body weight by multiplying the rates per number of cells as indicated in Fig. 2 by the number of

parenchymal cells per g of intact liver and by the liver weight per 100g body weight. The number of parenchymal cells per g of intact liver as determined from the activities of marker enzymes for parenchymal cells in homogenates from whole liver and from parenchymal cells was $0.80(\pm 0.03) \times 10^8$ cells/g ($n = 27$) and $1.29(\pm 0.05) \times 10^8$ cells/g ($n = 16$) for hepatocytes from fed and starved rats respectively ($P < 0.001$). The corresponding values for liver weight per 100g body weight were 4.40 ± 0.07 g ($n = 11$) and 3.38 ± 0.07 g ($n = 10$; $P < 0.001$). Glycerol 3-phosphate was expressed as $\mu\text{mol/g}$ of intact liver by multiplying its content per number of cells (Fig. 1) with the number of parenchymal cells per g of intact liver. Fig. 5 shows a shift to the right of the curve obtained for the starved state relative to that for the fed state. The 95% confidence intervals overlap over the whole course of the curves, indicating that differences are not statistically significant. However, the 90% confidence intervals do not overlap between 0.15 and $0.30 \mu\text{mol}$ of glycerol 3-phosphate/g of liver (not shown), suggesting that for a given fatty acid supply and glycerol 3-phosphate content below $0.3 \mu\text{mol/g}$ the liver may esterify less in the starved than in the fed state. This relative decrease in esterification in the starved state is likely the result of the decrease in glycerophosphate acyltransferase activity.

In the next series of experiments the glycerol 3-phosphate content of freeze-clamped livers *in vivo* was determined for fed and starved rats. The respective values were $0.305 \pm 0.030 \mu\text{mol/g}$ (fed, $n = 14$) and $0.443 \pm 0.077 \mu\text{mol/g}$ (starved, $n = 20$). Comparison of these values with the curves of Fig. 5 indicates that, as far as glycerol 3-phosphate availability *in vivo* is concerned, livers from fed and starved animals are capable of esterifying almost equal amounts of fatty acids, so that glycerol 3-phosphate availability itself does not seem to be responsible for a relative decrease in esterification in the starved state. For a fatty acid load comparable with that used in our experiments *in vitro*, livers from fed and starved animals would esterify *in vivo* 1.58 and $1.63 \mu\text{mol}$ of fatty acid/min per 100g body wt.

To ascertain that the above extrapolation yielded meaningful results, we determined the rate of triacylglycerol secretion into the circulation in the rat *in vivo*. Starved rats were used because they have high plasma concentrations of non-esterified fatty acids and because the triacylglycerols appearing in their plasma are largely of hepatic origin; $1.25 \pm 0.14 \mu\text{mol}$ of fatty acid/min per 100g body wt. ($n = 5$) was secreted in triacylglycerols into their circulation. Their plasma non-esterified fatty acid concentration was $0.81 \pm 0.20 \text{ mM}$, resulting in a fatty acid/albumin molar ratio of approx. 1.2, whereas under our experimental conditions *in vitro* a

Fig. 5. Glycerol 3-phosphate dependence of triacylglycerol synthesis *in vivo* as obtained by extrapolation of the experimental data *in vitro*

The experimental glycerol 3-phosphate contents, expressed as $\mu\text{mol}/10^8$ cells in Figs. 1 and 2, were converted into $\mu\text{mol/g}$ of intact liver by multiplication by the number of parenchymal cells per g of liver. The esterification rates, expressed as μmol of palmitate incorporated into triacylglycerol/min per 10^8 cells of Fig. 2, were converted into $\mu\text{mol/min}$ per 100g body wt by multiplication by the number of parenchymal cells per g of liver and by the liver weight expressed as g per 100g body weight. The broken curves represent the 95% confidence limits calculated as described in the Experimental section.

palmitate/albumin ratio of 2.3 was used. Given the differences in non-esterified fatty acid/albumin ratios and in non-esterified fatty acid composition, the data nevertheless show that the extrapolation yielded rates comparable with those observed *in vivo*.

Finally it should be noted that freshly isolated hepatocytes from fed rats have a glycerol 3-phosphate content comparable with that of the corresponding freeze-clamped livers. However, glycerol 3-phosphate in isolated hepatocytes from starved rats is 2–3-fold lower than in the corresponding freeze-clamped livers. The glycerol 3-phosphate content of 0.8×10^8 cells from fed animals (1g of intact liver) is $0.4 \mu\text{mol}/10^8$ cells (see above) $\times 0.8 = 0.32 \mu\text{mol}$; for 1.29×10^8 cells from starved animals (1g of intact liver) this value is $0.15 \mu\text{mol}/10^8$ cells (see above) $\times 1.29 = 0.19 \mu\text{mol}$.

Conclusions

Integrating the above observations, one can conclude that in isolated hepatocytes rates of triacylglycerol synthesis are primarily regulated by substrate supply. The intracellular acyl-CoA contents are determined by the supply of exogenous fatty acids and by the competing activity of the oxidation pathway, which is increased in starvation. However, at high external non-esterified fatty acid concentrations, oxidation rates become equally high in hepatocytes from fed and starved rats. In hepatocytes from starved animals glycerol 3-phosphate limits esterification, but its concentration can easily be raised by the addition of precursors. At saturating intracellular glycerol 3-phosphate concentrations, the esterification capacity is equal in both nutritional states. At subsaturating concentrations hepatocytes from starved rats esterify less as a result of the decrease in activity of glycerophosphate acyltransferase. A similar picture whereby triacylglycerol synthesis is primarily dictated by the concentrations of acyl-CoA and glycerol 3-phosphate seems to hold *in vivo*. At the glycerol 3-phosphate contents found *in vivo*, livers from fed and starved animals appear to be capable of esterifying the same amount of fatty acids. However, dietary or hormonal variations in glycerol 3-phosphate content in the range below $0.4 \mu\text{mol/g}$ for livers from fed animals and below $0.6 \mu\text{mol/g}$ for starved animals should cause large changes in esterification.

When triacylglycerol entry into the circulation is measured *in vivo*, fed and starved rats secrete comparable amounts of triacylglycerols (results not shown). Since part of the triacylglycerols is undoubtedly of intestinal origin in the fed state, these observations suggest that in starvation the liver actually esterifies more and secretes more very-low-density lipoprotein than in the fed state, most probably as a result of the high non-esterified fatty acid influx from adipose tissue.

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