The Influence of Ethanol-induced Changes of the

α-Glycerophosphate Level on Hepatic Triglyceride Synthesis

ERIK FELLENIUS, GUNNAR BENGTSSON and
KARL-HEINZ KIESSLING

Alcohol Research Group of the Swedish Medical Research Council, Institute of
Zoophysiology, University of Uppsala, Uppsala, Sweden

The correlation between the level of α-glycerophosphate and hepatic triglyceride synthesis was studied in rat liver. Livers were perfused *in situ* with and without oleate in the medium and the hepatic content of α-glycerophosphate was varied by using livers of rats in different nutritional states and by adding ethanol, pyruvate or glycerol to the perfusate.

It was observed that changes in triglycerides were not a function of the concentration of α-glycerophosphate in the absence of oleate. In the presence of oleate, alone or with other substrates, a correlation could be observed. Pyruvate tended to inhibit the ethanol-induced increase of α-glycerophosphate and malate. Oleate also decreased the ethanol-induced accumulation of α-glycerophosphate in the livers of starved rats but not in those of rats on normal diet.

The importance of the availability of proton acceptors for the regulation of the level of α-glycerophosphate is discussed. The capacity of the isolated liver mitochondria of rats fed a high-fat diet to oxidize α-glycerophosphate was twice as great as that of the liver mitochondria of rats on a normal diet. The result indicates a relationship between lipid metabolism and the activity of the mitochondrial α-glycerophosphate dehydrogenase.

Two major metabolic pathways are available to the free fatty acids entering the liver: catabolism *via* β-oxidation or esterification with α-glycerophosphate to form triglycerides. Obviously, the higher the proportion of free fatty acids which are converted into triglycerides, the less there is available for oxidation to acetyl-CoA. It has been suggested that the main factor controlling the intrahepatic fate of free fatty acids is the availability of α-glycerophosphate.[1-6] Indeed Howard and Lowenstein [7] presented evidence that the level of α-glycerophosphate might control the *de novo* synthesis of hepatic fatty acids. In certain nutritional states, fatty acid synthesized *de novo* may be a substrate of quantitative importance for triglyceride synthesis.[8]

Studies of the flux of carbon atoms from labelled glucose to glyceride-glycerol in hearts throw serious doubt on the assumption discussed above.[9,10]

A lack of correlation between the ability of various compounds to reverse starvation ketosis in the rat liver and their effects on the α-glycerophosphate concentration in the liver has also been reported.[11],[12]

The purpose of the study reported here is to investigate quantitative relationship between the hepatic level of α-glycerophosphate and the triglyceride synthesis. For this purpose, livers were perfused *in situ* with or without equimolar quantities of oleate. The hepatic content of α-glycerophosphate was varied by the perfusion of the livers of rats in different nutritional states and by the addition of ethanol, pyruvate or glycerol. It is obvious from the results that net decrease of triglycerides in the liver is not a function of the level of α-glycerophosphate. However, during the net synthesis of neutral fat, a correlation was found between the changes in triglyceride content and the level of α-glycerophosphate at the end of the experiment. The factors affecting the level of α-glycerophosphate are discussed.

EXPERIMENTAL

Rats. Female Wistar rats (180 – 230 g) from the laboratory's stock were used. The animals were divided into fed, 48 h starved, and fat-fed groups as described in Ref. 13.

Reagents. Standard analytical-grade laboratory reagents were obtained from E. Merck AG, Darmstadt, West Germany. Oleic acid *(puriss.)* was obtained from Fluka AG, Chemische Fabrik, Buchs, Switzerland. All the enzymes and co-enzymes were obtained from Biochimica Boehringer, Mannheim, West Germany, or Sigma Chemical Co., St Louis, USA.

Liver perfusion and addition of agents. The method of liver perfusion was that described by Hems *et al.*,[14] except that fresh bovine erythrocytes, supplied by the slaughterhouse at Uppsala, Sweden, was used instead of aged human erythrocytes.

Substrates were added to the medium either as a single dose (ethanol, oleate) or by continuous infusion (glycerol, pyruvate) beginning 38 min after the start of the perfusion. Oleic acid was added as a neutral solution bound to albumin.[15] Before addition of substrates, one lobe of the liver was freeze-clamped.[16] The perfusion was continued for 45 min after the initial addition of substrate. At that time the rest of the liver was freeze-clamped. In the tables 0 min refer to the time of the first sampling of the liver, namely after 38 min of perfusion. Liver and perfusate were deproteinized with $HClO_4$ and were prepared for metabolite assays as described by Williamson *et al.*[17]

Warburg experiments. The rats on a normal diet and rats fed a high-fat diet were decapitated and the liver mitochondria were prepared for manometric studies according to the method of Ernster and Löw.[18] The mitochondria were suspended in sucrose (0.25 M), so that the 1 ml added to the Warburg vessel contained mitochondria from about 300 mg of liver.

The final concentration of substances in the incubation mixture was K-phosphate buffer, pH 7.4, 15.0 mM; ATP, 2.0 mM; $MgCl_2$, 5.0 mM; glucose, 25.0 mM; DL-α-glycerophosphate, 15.0 mM; hexokinase, 30 units.[19] The respiratory rate with DL-α-glycerophosphate (Sigma Chemical Co., St. Louis, USA) was determined manometrically. The rate of disappearance of α-glycerophosphate was also measured. Protein was estimated according to Lowry *et al.*[20]

Determination of metabolites. Lactate, malate, and α-glycerophosphate were determined by the method of Hohorst *et al.*[21] Triglycerides were estimated by a modification of the method described by Eggstein and Kreutz.[22]

The freeze-clamped liver tissue was ground in a cooled percussion mortar.[16] About 0.1 g of liver was transferred to a tube containing 2.0 ml of 0.50 M KOH dissolved in ethanol and previously weighed and chilled in liquid nitrogen. The tube was allowed to thaw and was weighed again. Hydrolysis of triglyceride was performed on a water bath (65 – 75°) for at least 1 h. Six millilitres of 0.10 M $MgSO_4$ were added and the solution was

chilled to 4°. After centrifugation for 15 min at 3000 g_{av}, the clear, neutralized supernatant was analyzed for its content of free glycerol (equivalent to the molar content of triglyceride) by the enzymatic method described by Eggstein and Kreutz.[22]

RESULTS

Changes of liver triglycerides. Hepatic triglyceride concentrations (Table 1) decreased significantly in starved, but not in fed animals during perfusion in the absence of oleate. While ethanol or pyruvate had no influence at all, a

Table 1. Changes of triglycerides in the perfused liver. Before the addition of substrates, one lobe of the liver was freeze-clamped. Thereafter, different substrates were added to the medium, either as a single dose (ethanol and oleate, final concentration 10 and 2 mM, respectively) or by continuous infusion of a 1.93 M pyruvate or 0.33 M glycerol solution at a rate of 1.83 ml/h. The livers were perfused for 45 min after the addition of substrates. The infusion of pyruvate was stopped after 40 min. At the end of the experiment, the rest of the liver was freeze-clamped. The rates are means $\pm$ S.E.M. for the number of observations stated in the parentheses.

Substrate added	Changes of liver triglycerides μmol/ (45 min g wet wt)		
	Normal diet	Starved for 48 h	High-fat diet
None	$-0.14 \pm 0.41(7)$	$-3.47 \pm 0.53(7)$	
Ethanol	$+0.09 \pm 0.43(9)$	$-2.43 \pm 0.69(7)$	
Oleate	$+4.74 \pm 0.29(9)$	$-0.37 \pm 0.60(9)$	
Ethanol + oleate	$+7.50 \pm 0.33(9)$	$+1.30 \pm 0.48(7)$	$-1.62 \pm 0.73(5)$
Pyruvate		$-3.99 \pm 0.87(6)$	
Pyruvate + ethanol		$+0.16 \pm 0.51(6)$	
Pyruvate + oleate		$-1.63 \pm 0.73(7)$	
Pyruvate + ethanol + oleate		$+3.88 \pm 0.74(8)$	
Glycerol + oleate		$+4.44 \pm 0.67(9)$	

net breakdown of triglyceride was eliminated in starved animals in the presence of oleate. A net increase of triglycerides could be demonstrated in the presence of oleate in the liver of normal diet rats. This increment was further augmented when ethanol was also present.

Correlation between changes of triglycerides and the level of α-glycerophosphate. When oleate was present, with or without other substrates, a correlation was found between the changes of triglycerides and the level of α-glycerophosphate (Fig. 1). The highest rate of triglyceride synthesis could be demonstrated in liver from normal-diet rats in the presence of ethanol and oleate. These tissues also had a very high level of α-glycerophosphate. The curve relating α-glycerophosphate concentration and net triglyceride synthesis tended to level off at high α-glycerophosphate concentrations possibly due to saturation of the enzyme system responsible for triglyceride synthesis. Whether the results from the experiments with glycerol and oleate represent such a saturation remains to be explored. It is noteworthy that the levels of α-glycerophosphate obtained in the present study were unphysiological and it is possible that

Table 2. The level of α-glycerophosphate, lactate and malate in the perfused liver. The experimental conditions were as described in Table 1. The values refer to the time of the addition of substrates (0 min) and after perfusion for 45 min. The values are means ± S.E.M. for the number of perfusions stated and are expressed as $\mu mol/(g$ wet wt).

| Animal treatment	Substrate added	Number of observations	α-Glycerophosphate		Lactate		Malate	
			0 min	45 min	0 min	45 min	0 min	45 min
Normal diet	None	7	0.230±0.038	0.307±0.021	1.69 ±0.16	1.78 ±0.18	0.435±0.033	0.474±0.091
	Ethanol	9	0.303±0.050	0.652±0.055	1.61 ±0.11	1.94 ±0.33	0.356±0.039	0.517±0.068
	Oleate	9	0.339±0.043	0.385±0.044	1.61 ±0.27	0.58 ±0.11	0.461±0.051	0.890±0.083
	Ethanol + oleate	9	0.333±0.028	0.783±0.079	1.72 ±0.15	1.14 ±0.13	0.384±0.021	0.930±0.114
	None	7	0.091±0.021	0.092±0.024	0.267±0.031	0.211±0.024	0.077±0.016	0.069±0.013
	Ethanol	7	0.080±0.020	0.886±0.068	0.217±0.041	0.355±0.059	0.053±0.010	0.173±0.016
	Oleate	9	0.049±0.004	0.173±0.021	0.199±0.010	0.257±0.037	0.064±0.007	0.149±0.013
	Ethanol + oleate	7	0.078±0.009	0.124±0.014	0.202±0.028	0.429±0.031	0.083±0.011	0.156±0.022
Starved for 48 h	Pyruvate	6	0.061±0.010	0.053±0.001	0.184±0.018	2.67 ±0.15	0.077±0.020	0.657±0.030
	Oleate + pyruvate	7	0.085±0.009	0.120±0.010	0.228±0.004	3.85 ±0.16	0.73 ±0.006	1.64 ±0.07
	Ethanol + pyruvate	6	0.069±0.006	0.123±0.014	0.268±0.041	4.45 ±0.31	0.060±0.012	0.699±0.039
	Ethanol + oleate + pyruvate	8	0.106±0.017	0.319±0.031	0.215±0.047	3.49 ±0.35	0.094±0.035	2.00 ±0.15
	Glycerol + oleate	9	0.086±0.023	7.56 ±0.51	0.200±0.011	0.232±0.024	0.078±0.011	0.185±0.012
High-fat diet	Ethanol + oleate	5	0.128±0.027	0.155±0.018	0.308±0.054	0.940±0.089	—	—
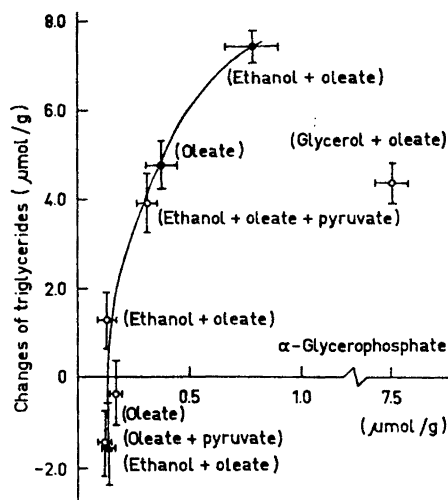
Fig. 1. Correlation between changes of hepatic triglycerides and the level of α-glycerophosphate. Livers from starved rats (O), rats on a normal diet (●) and rats on a high-fat diet (▲) were perfused as described in Table 1. The changes of hepatic triglycerides during 45 min in the presence of oleate have been plotted against the level of α-glycerophosphate after perfusion for 45 min. The values are means ± S.E.M. for 6 − 9 perfusions.

other factors were regulatory under these experimental conditions. Nevertheless, the results in those instances in which the amount of α-glycerophosphate varied within the physiological range, are consistent with the hypothesis that the concentration of this compound is a physiological regulator of the synthesis of neutral fat.

In livers perfused with a medium free of oleate, hepatic triglyceride stores either decreased or were unchanged (Table 1); no correlation was found between the magnitude of the change in triglyceride and the concentration of α-glycerophosphate.

Factors affecting the level of α-glycerophosphate. The level of α-glycerophosphate, malate, and lactate will depend to a large extent on the cytoplasmic [free NAD_+]/[free NADH] ratio.[21] One of the most characteristic events occurring in the liver during the oxidation of ethanol is a dramatic increase of this redox ratio.[23-26] Therefore, in order to investigate how the three compounds are interrelated during ethanol metabolism, an analysis was made of the content of α-glycerophosphate, malate, and lactate (Table 2).

In livers from starved and normal diet rats, the contents of α-glycerophosphate and malate increased in the presence of ethanol, while the level of lactate was less affected. Oleate decreased the lactate level, an effect which was slightly inhibited by ethanol. Oleate alone did not influence the concentration of α-glycerophosphate appreciably. During ethanol oxidation, however, oleate prevented the accumulation of α-glycerophosphate in the livers of starved rats but not in the livers of rats on a normal diet. Surprisingly, the oleate-induced accumulation of malate was not further increased by ethanol. When pyruvate was infused in the presence of ethanol, the amount of lactate increased while α-glycerophosphate and malate did not change appreaciably. Thus a considerable portion of the reducing equivalents derived from the oxidation of ethanol was transferred to pyruvate. Since pyruvate acts as gluconeogenetic precursor the NADH will also be consumed along this path-

way. In consequence, dihydroxyacetone phosphate and oxaloacetate will be ruled out as the proton acceptors.

Increased capacity of the mitochondria to oxidize α-glycerophosphate. The rate of disappearance of α-glycerophosphate and the uptake of oxygen by isolated mitochondria obtained from the livers of fat-fed and normal diet animals in Warburg experiments are presented in Table 3. The capacity to

Table 3. Induction of the liver mitochondria of rats fed a high-fat diet to oxidize α-glycerophosphate, as measured in Warburg experiments. Details of the experimental conditions have been given in the "Experimental" section. The values are means ± S.E.M. for the number of animals stated. The rates are expressed as μmol/(h 10 mg protein) and the concentrations of triglycerides as μmol/(g wet wt).

Animal treatment	Number of observations	Oxygen uptake	α-Glycero- phosphate consumption	Triglyceride content
Normal diet	6	7.2 ± 0.4	6.6 ± 0.4	11.7 ± 0.2
High-fat diet	6	19.5 ± 1.6	15.9 ± 1.0	52.2 ± 4.9

oxidize α-glycerophosphate was more than twice as great in the livers of rats on high-fat diets, compared with normal livers. The level of triglyceride was about five times higher in rats fed a high-fat diet compared with normal diet rats. The results suggest a relationship between the rate of triglyceride synthesis and the activity of the mitochondrial α-glycerophosphate dehydrogenase.

DISCUSSION

The results (Fig. 1) suggest that the concentration of α-glycerophosphate should be considered as one of the factors, but not the only one, influencing the hepatic triglyceride biosynthesis. In perfusions carried out in the absence of oleate, no correlation between the level of α-glycerophosphate and the rate of triglyceride synthesis was found. These were experiments in which the levels of triglycerides either decreased or were unchanged. In this instance the availability of fatty acid for triglyceride synthesis was probably rate limiting.

At very high, unphysiological levels of α-glycerophosphate, brought about by the infusion of glycerol, only a moderate increase in hepatic triglycerides was found in spite of the presence of oleate. Other factors were obviously rate limiting in this experiment.

Since it was found that the level of α-glycerophosphate may be a regulator of net triglyceride synthesis it is also of interest to know what factors influence the concentration of α-glycerophosphate. This may be the flux of α-glycerophosphate into mitochondria,[27] the supply from glycolysis, gluconeogenesis, lipid metabolism, the pentosphosphate pathway, the kinetic behaviour of α-glycerophosphate dehydrogenase[28,29] in the intact animal, the uptake of

glycerol from the blood and finally the redox state [21],[30] or the availability of different proton acceptors.[31]

The NADH formed during ethanol oxidation may be utilized by the glyceraldehyde phosphate, lactate, malate, or α-glycerophosphate dehydrogenase systems, disregarding other biosynthetic or hydroxylation reactions which may directly utilize the NADH produced in the cell sap. Thus information on the interrelationship between α-glycerophosphate, malate, and lactate is of importance for a knowledge of the role of the availability of proton acceptors. It is clear from the results (Table 2) that the availability of pyruvate for different metabolic pathways is limited during ethanol oxidation. Lactate represents a metabolic end product and it would have increased more if the conversion of pyruvate to lactate was the main route of hydrogen transfer. Moreover, a net decrease of lactate was found with normal diet rats when oleate or oleate and ethanol had been added. Thus, hydrogen is mainly transferred to oxaloacetate and dihydroxyacetone phosphate during ethanol oxidation. As a consequence the corresponding reduced compounds, malate and α-glycerophosphate, increased. The powerful inhibition by α-glycerophosphate of α-glycerophosphate dehydrogenase [28],[29] will prevent an indefinite accumulation of α-glycerophosphate. The suggested route for hydrogen transfer is further supported by the results when pyruvate was infused. Then, pyruvate availability is not the limiting factor and as a consequence the ethanol-induced rise in malate and α-glycerophosphate formation is prevented.

The importance of enzyme(s) located within the mitochondria on the level of α-glycerophosphate is not quite established. The activity of the flavin-dependent α-glycerophosphate dehydrogenase (EC 1.1.99.5) is considered to be too low to be of any significance.[32–34] In a recent report [35] it was shown that the rate of oxidation of α-glycerophosphate by the intact organ exceeded by far the calculated capabilities of isolated mitochondria. We found that liver mitochondria from fat-fed rats had an increased capacity to oxidize α-glycerophosphate (Table 3). The net sequence of this would be to limit the accumulation of α-glycerophosphate in the liver and in this way to inhibit the synthesis of triglycerides. A similar adaptation occurs after long-term feeding with ethanol.[36],[37] Thus, there seems to exist a relationship between the relative disposition of fatty acid into the β-oxidative and triglyceride synthetic pathways and the activity of a mitochondrial enzyme, the flavin-dependent α-glycerophosphate dehydrogenase, which will influence the level of α-glycerophosphate.

Carnicero et al.[35] also presented evidence that the pathway by which extramitochondrial hydrogen in form of α-glycerophosphate is oxidized partly leads through a respiratory chain-linked NADH-dehydrogenase. Part of the disappearance of α-glycerophosphate may therefore be due to interaction between this dehydrogenase and other dehydrogenases as malate, β-hydroxybutyrate, or glutamate dehydrogenase within the mitochondria. Thus the small increase of α-glycerophosphate in starved and fat-fed rats supplemented with oleate and ethanol but not in fed rats (Table 2) can partly be explained by the higher rate of acetoacetate formation in the former group. The hydrogen formed during the oxidation of α-glycerophosphate will be reoxidized by the β-hydroxybutyrate dehydrogenase system in the livers of starved and fat-fed

rats but not in livers from normal diet rats, where the rate of ketone-body formation is fairly low. It should be noted that, when oleate was present, ethanol did not induce any accumulation of malate. Possibly the β-hydroxy-butyrate dehydrogenase is more important than the malate dehydrogenase for the reoxidation of α-glycerophosphate under the present experimental conditions.

REFERENCES

1. Fritz, I. B. *Physiol. Rev.* **41** (1961) 52.
2. Wieland, O. and Matschinsky, F. *Life Sci.* **1** (1962) 49.
3. Nikkilä, E. A. and Ojala, K. *Proc. Soc. Exp. Biol. Med.* **113** (1963) 814.
4. Nikkilä, E. A. and Ojala, K. *Life Sci.* **10** (1963) 717.
5. Tzur, R., Tal.E. and Shapiro, B. *Biochim. Biophys. Acta* **84** (1964) 18.
6. Ylikahri, R. H. *Metabolism* **19** (1970) 1036.
7. Howard, C. F., Jr. and Lowenstein, J. M. *J. Biol. Chem.* **240** (1965) 4170.
8. Windmueller, H. G. and Spaeth, A. E. *Arch. Biochem. Biophys.* **122** (1967) 362.
9. Denton, D. M. and Randle, P. J. *Biochem. J.* **104** (1967) 423.
10. Denton, D. M. and Halperin, M. L. *Biochem. J.* **110** (1968) 27.
11. Williamson, D. H., Veloso, D., Ellington, E. V. and Krebs, H. A. *Biochem. J.* **114** (1969) 575.
12. McGarry, J. D. and Foster, D. W. *J. Biol. Chem.* **246** (1971) 6247.
13. Fellenius, E. and Kiessling, K.-H. *Acta Chem. Scand.* **27** (1973) 2781.
14. Hems, R., Ross, B. D., Berry, M. N. and Krebs, H. A. *Biochem. J.* **101** (1966) 284.
15. Krebs, H. A., Wallace, P. G., Hems, R. and Freedland, R. A. *Biochem. J.* **112** (1969) 595.
16. Wollenberger, A., Ristau, O. and Schoffa, G. *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270** (1960) 399.
17. Williamson, D. H., Lund, P. and Krebs, H. A. *Biochem. J.* **103** (1967) 514.
18. Ernster, L. and Löw, H. *Exptl. Cell Res.,* Suppl. **3** (1955) 113.
19. Darrow, R. A. and Colowick, S. P. *Methods Enzymol.* Academic, New York and London (1962), p. 226.
20. Lowry, O. H., Rosebrough, N. H., Farr, A. L. and Randell, R. J. *J. Biol. Chem.* **193** (1951) 265.
21. Hohorst, H. J., Kreutz, F. H. and Bücher, Th. *Biochem. Z.* **332** (1959) 18.
22. Eggstein, M. and Kreutz, F. H. *Klin. Wschr.* **44** (1966) 262.
23. Forsander, O., Räihä, N. and Suomalainen, H. *Hoppe-Seyler's Z. physiol. Chem.* **312** (1958) 243.
24. Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G. and Fukami, M. H. *J. Biol. Chem.* **244** (1969) 5044.
25. Lindros, K. O. and Aro, H. *Ann. Med. Exp. Fenn.* **47** (1969) 39.
26. Rawat, A. K. *Eur. J. Biochem.* **6** (1968) 585.
27. Lehninger, A. L. *The Mitochondrion,* Benjamin, New York 1964, p. 132.
28. Blanchaer, M. C. *Can. J. Biochem.* **43** (1965) 17.
29. Black, W. J. *Can. J. Biochem.* **44** (1966) 1301.
30. Veech, R. L., Guynn, R. and Veloso, D. *Biochem. J.* **127** (1972) 387.
31. Schimassek, H., Walli, A. K. and Höfer, G. In Martini, G. A. and Bode, Ch., Eds., *Metabolic Changes Induced by Alcohol,* Springer, Berlin, Heidelberg, New York 1971, p. 157.
32. Kleitke, B., Heier, G. and Wollenberger, A. *Biochim. Biophys. Acta* **130** (1966) 270.

33. Klingenberg, M. *Eur. J. Biochem.* **13** (1970) 247.
34. Hassinen, I., Ylikahri, R. and Kähönen, M. *Ann. Med. Exp. Fenn.* **48** (1970) 176.
35. Carnicero, H. H., Moore, C. L. and Hoberman, H. D. *J. Biol. Chem.* **247** (1972) 418.
36. Kiessling, K.-H. *Acta Pharmacol. Toxicol.* **26** (1968) 245.
37. Rawat, A. K. and Kyriyama, K. *Biochem. Biophys. Res. Commun.* **47** (1972) 517.