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Effect of Gluconeogenic Substrates on Ketogenesis in Isolated Rat Hepatocytes. I. Effect of Glycerol *

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The effect of glycerol on the fate of free fatty acids in isolated hepatocytes from starved rats has been studied. 5 mM glycerol inhibited the ketone bodies production from endogenous substrates, acetate, octanoate or oleate. The incorporation of radioactivity into ketone bodies from acetate, octanoate or oleate was also inhibited by glycerol. The oxidation of radioactive fatty acids to CO_2 was not significantly changed by glycerol. However, the incorporation of acetate, octanoate or oleate carbons into liver lipids were increased by glycerol.

Since EDSON (7) described in 1936 the inhibition of acetoacetate production caused by glycerol, the antiketogenic effect of this compound has been profusely studied. Briefly, the effect of glycerol on ketogenesis has been explained through, the increase of α-glycerolphosphate concentration which enhanced fatty acid esterification (8, 20); the enhancement of glycolysis which resulted on the decrease of the oxidative phosphorylation by competition for ADP (19). This fact might promote the decrease of the rate of respiratory chain which prevented the recuperation of oxidized coenzymes for β-oxidation, and the enhancement of the rate of the tricarboxilic acid cycle as a consequence of the decrease of ATP (4, 23) and/or the increase of oxaloacetate concentration (10). However, none of these hypotheses have been definitively proved.

The present investigation was designed to study the effect of glycerol on the fate of free fatty acids in isolated hepatocytes from starved rats. Consequently, the effect of glycerol on the ketone body production and on the incorporation of the radioactivity into ketone bodies, CO_2 and

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liver lipids from acetate, octanoate and oleate has been investigated.

Materials and Methods

Reagents. Substrates, enzymes and coenzymes were obtained from Sigma Chemical Co. Boehringer or Serva Feinbiochemical. Standard analytical-grade laboratory reagents were purchased from Sigma or Merck. Bovine serum albumin was obtained from Armour Pharmaceutical Co. Sodium pentobarbital was a gift from Abbot Laboratories. 1-^{14}C-acetate, 1-^{14}C-octanoate and 1-^{14}C-oleate were obtained from The Radiochemical Centre.

Animals. Female albino Wistar rats, fed on a stock laboratory diet, were used for experiments between 9 and 10 a.m. Animals were deprived of food 48 h before the experiments but were allowed to take water *ad libitum.*

Isolation and incubation of hepatocytes. Hepatocytes were prepared by the method of BERRY and FRIEND (2) with the modifications described by CORNELL *et al.* (5). Rat livers were perfused with calcium-free Krebs-Henseleit physiological saline containing colagenase and hyaluronidase (0.03 % w/v of each). After 20 min the liver was scissed and incubated in the same medium during 10 min. The preparation was filtered through a nylon mesh (0.44 × 0.44 mm) and washed twice with Krebs-Henseleit physiological saline (11) containing dialysed bovine serum albumin (2.5 % w/v).

Analytical methods. Acetoacetate and 3-hydroxybutyrate were assayed as described by WILLIAMSON *et al.* (22) and long-chain acyl-CoA according to MICHAL and BERMEYER (17). Labeled ketone bodies were isolated as acetone-mercurial complex as described by BATES *et al.* (1). Radioactivity found in the carbonylic carbon of acetoacetate was considered as one half of the total. The asymetry labeling of acetoacetate was not changed by the presence of glycerol (P. Rosario, unpublished results). Radioactivity in 3-hydroxybutyrate was calculated according to the 3-hydroxybutyrate/acetoacetate ratio measured spectrophotometrically. Radioactivity in CO_2 was measured by trapping in 1 N NaOH the CO_2 evolved after acidification of the incubation medium with 60 % (w/v) perchloric acid. Radioactivity in liver lipids was measured in the washed chloroform-methanol (2 : 1) liver cell extracts (6). These fractions are considered as «liver lipids» which include triacylglycerols, phospholipids, cholesterol and cholesterol-esters.

Results

Table I shows the effect of glycerol on the ketone body production by isolated hepatocytes from starved rats. 5 mM glycerol inhibited ketone body production from endogenous substrates, acetate, octanoacetate or oleate. Glycerol decreased the production of both species of ketone body but 3-hydroxybutyrate production from acetate was not changed by glycerol. This was not unexpected considering that acetate *per se* depressed the 3-hydroxybutyrate synthesis. It is noteworthy that octanoate and oleate but not acetate increased the 3-hydroxybutyrate/acetoacetate ratio suggesting that 3-hydroxybutyrate synthesis depends on the reducing equivalents coming from β-oxidation.

Table II shows the effect of glycerol on the fate of radioactivity from labeled acetate, octanoate or oleate in isolated hepatocytes from starved rats. 5 mM glycerol inhibited the incorpation of acetate, octanoate and oleate carbons into ketone bodies. However, the effect of glycerol was not observed in the experiment in which only labeled acetate was added. This fact suggests that the conversion of acetate into ketone bodies requires high concentra-

Table I. *Effect of glycerol on ketone bodies production from endogenous substrates, acetate, octanoate or oleate.*
Hepatocytes coming from starved (48 h) rats were incubated for 1 h. Results are expressed in μmolcs/h/g wet cells and are means ± S.E.M. (n = 6-8).

Additions to the incubation medium	Acetoacetate	3-hydroxybutyrate	Ketone bodies	3-hydroxybutyrate/ acetoacetate
none	38 ± 1	18 ± 1	56 ± 1	0.66
5 mM glycerol	21 ± 1 **	8 ± 2 **	29 ± 2 **	0.38
5 mM acetate	37 ± 2	6 ± 2	43 ± 2	0.18
5 mM acetate + 5 mM glycerol	21 ± 1 **	7 ± 2	28 ± 2 **	0.32
2 mM octanoate	33 ± 3	60 ± 3	93 ± 5	1.73
2 mM octanoate + 5 mM glycerol	12 ± 1 **	10 ± 1 **	22 ± 1 **	0.84
2 mM oleate	39 ± 1	52 ± 2	91 ± 3	1.05
2 mM oleate + 5 mM glycerol	25 ± 1 **	32 ± 1 **	57 ± 1 **	1.20

** $p < 0.01$.

Table II. *Effect of glycerol on the fate of free fatty acids in isolated rat hepatocytes.* Hepatocytes coming from starved rats were incubated for 1 h in the presence of 10 μCi of the radioactive fatty acid per gram of wet cells. Results are expressed in d.p.m. per g of wet cells × 10^{-3} and are means ± S.E.M. (n = 6-8).

Additions to the incubation medium	Liver lipids	CO_2	Ketone bodies
1-^{14}C acetate	131 ± 11	1.021 ± 38	115 ± 15
1-^{14}C acetate + 5 mM glycerol	213 ± 13 **	905 ± 20	123 ± 6
1-^{14}C acetate + 5 mM acetate	42 ± 9	653 ± 72	273 ± 10
1-^{14}C acetate + 5 mM acetate + 5 mM glycerol	130 ± 17 **	774 ± 90	138 ± 21 **
1-^{14}C octanoate + 2 mM octanoate	195 ± 33	1.423 ± 120	263 ± 20
1-^{14}C octanoate + 2 mM octanoate + 5 mM glycerol	567 ± 25 **	1.768 ± 170	218 ± 8 *
1-^{14}C oleate + 2 mM oleate	1.393 ± 161	608 ± 90	214 ± 30
1-^{14}C oleate + 2 mM oleate + 5 mM glycerol	3.065 ± 903 **	517 ± 62	122 ± 10 **

* $p < 0.1$; ** $p < 0.01$.

tions of acetate to be competitive with endogenous fatty acids. In addition, acetate increased *per se* the incorporation of radioactivity into ketone bodies but decreased the label in liver lipids. This result suggests that the increase of acetate concentrations decreases the cytosolic utilization of acetyl-CoA for lipogenesis.

On the other hand glycerol increased the incorporation of acetate, octanoate and oleate carbons into liver lipids (table II). This effect was also observed in the experiment in which only labeled acetate was added. However the incorporation of radioactive fatty acids into CO_2 was not significantly changed by glycerol.

Table III shows the long-chain acyl-CoA concentrations in hepatocytes incubated in the experimental conditions used for the experiments depicted in tables I and II. The presence of acetate or oleate in the incubation medium increased the long-chain acyl-CoA concentration in the liver cells. The effect of oleate is not unexpected because oleate may be a direct source of long-chain acyl-CoA. However, the effect of acetate increasing long-chain acyl-CoA concentration is striking and presumably indirect. Although this fact requires a further investigation, it may be suggested that acetate decreases the long-chain fatty acids utilization by competition with β-oxidation for coenzyme A. Thus, the depletion of coenzyme A presumably caused by acetate might prevent the conversion of long-chain fatty acids into acyl-CoA, a compulsory step for β-oxidation. On the other hand, 5 mM glycerol decreased long-chain acyl-CoA concentration in the presence of acetate or octanoate (table III).

Table III. *Effect of glycerol on long-chain acyl-CoA concentration in isolated rat hepatocytes.*

Hepatocytes coming from starved rats were incubated for 30 min. Results are expressed in μmol/g wet cells and are means ± S.E.M ($n = 6$-8).

Additions to the incubation medium	Long-chain acyl-CoA
none	4.9±0.5
5 mM glycerol	5.6±0.4
5 mM acetate	5.9±0.4
5 mM acetate + 5 mM glycerol	4.3±0.2 **
2 mM octanoate	4.9±0.4
2 mM octanoate + 5 mM glycerol	2.9±0.5 **
2 mM oleate	7.5±0.9
2 mM oleate + 5 mM glycerol	8.6±1.6

** $p < 0.01$.

Discussion

The antiketogenic effect of glycerol has been explained as a consequence of the enhancement of the rate of free-fatty acid esterification caused by the increase of α-glycerol phosphate concentrations (8,20). However, the effect of glycerol is also observed in the presence of octanoate (tables I and II) which is considered a non-esterifiable fatty acid (7, 12). Therefore, it may be assumed that glycerol interferes with ketogenesis in steps other than esterification. In addition, the antiketogenic effect of glycerol is also observed in the presence of acetate (tables I and II) a fact which suggests that glycerol does not affect carnitin-transferase activities (18, 24) and/or β-oxidation. Consequently, it may be concluded from our results that glycerol interferes with the fate of acetyl-CoA. Actually, it has been suggested that some antiketogenic agents inhibit ketogenesis by the enhancement of the rate of tricarboxylic acid cycle as a consequence of the increase of the oxalacetate concentrations (10, 13, 21). This is probably not the case with glycerol since no changes in the la-

beled CO_2 evolved were observed in the presence of glycerol (tables I and II). On the other hand, glycerol increased the radioactivity incorporated into liver lipids (table II), a fact consistent with the idea that glycerol increases the rate of lipogenesis *de novo*. Actually, although the experiments with oleate do not exclude the enhancement of esterification, the increase of acetate and octanoate incorporation into liver lipids clearly suggests that the presence of glycerol increased the rate of lipogenesis *de novo*. Regarding the experiments with oleate, it should be mentioned that the enhancement of lipogenesis caused by glycerol may increase the rate of oleate esterification through the rise of malonyl-CoA concentration. Thus, it has been suggested that malonyl-CoA concentration regulates the fate of long-chain acyl-CoA by the inhibition of palmitoyl-carnitine transferase activity (14-16). Consequently, a rise of malonyl-CoA concentration may inhibit fatty acid oxidation which in turn increases the rate of esterification.

Likewise, it may be suggested that the effect of glycerol on ketogenesis may be mediated by the stimulation of malonyl-CoA synthesis and/or the citrate efflux from mitochondria as the main limiting steps of lipogenesis. Actually, it has been reported that long-chain acyl-CoA inhibits acetyl-CoA carboxylase activity and mitochondrial citrate transporter (3, 9). If so, a decrease of long- chain acyl-CoA concentration as a consequence of the enhancement of acyl-glycerol synthesis caused by glycerol may decrease the rate of ketogenesis through the deinhibition of lipogenesis. Actually, glycerol decreased long-chain acyl-CoA concentration in the presence of acetate or octanoate (table III) but this effect was not observed in the presence of oleate or in the absence of exogenous fatty acids. However it should be pointed out that the proposed mechanism for the deinhibition of lipogenesis required changes in the long-chain acyl-CoA concentrations which may not be detectable with the available methods. Consequently, it may be assumed that in the presence of oleate or in the absence of exogenous fatty acids the changes of long-chain acyl-CoA pool which controls lipogenesis may be overlapped by the changes of the long-chain acyl-CoA concentrations which presumably occur in other cellular compartments in these circumstances.

In conclusion, our results suggest that glycerol inhibits ketogenesis by increasing the rate of lipogenesis *de novo*. Whether this effect is mediated by the decrease of long-chain acyl-CoA concentrations caused by glycerol which deinhibits the acetyl-CoA carboxylase and/or citrate transporter, or by other unknown mechanism remains to be dilucidated.

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Resumen

Se estudia el efecto del glicerol sobre el destino de los ácidos grasos en hepatocitos procedentes de ratas en ayuno. El glicerol 5 mM inhibe la producción de cuerpos cetónicos a partir de substratos endógenos, acetato, octanoato y oleato, así como la incorporación en cuerpos cetónicos de la radiactividad procedente de acetato, octanoato y oleato. La presencia de glicerol no modifica substancialmente la oxidación a CO_2 de los ácidos grasos radiactivos, aunque aumenta significativamente la incorporación de C^{14} procedentes de acetato, octanoato u oleato en lípidos hepáticos.

References

1. BATES, M. W., KREBS, H. A. and WILLIAMSON, D. H.: *Biochem. J.*, 110, 655-661, 1968.
2. BERRY, M. N. and FRIEND, D. S.: *J. Cell. Biol.*, 43, 506-520, 1969.

3. BORTZ, W. M. and LYNEN, F.: *Biochem. Z.*, **337**, 505-509, 1963.
4. BURCH, H. B., LOWRY, O. H., MEINHARDT, L., MAX, P. Jr. and CHYU, K.: *J. Biol. Chem.*, **245**, 2092-2102, 1970.
5. CORNELL, N. W., LUND, P., HEMS, R. and KREBS, H. A.: *Biochem. J.*, **134**, 671-672, 1973.
6. CUZNER, M. L. and DAVIDSON, A. N.: *J. Chromat.*, **27**, 388-397, 1967.
7. EDSON, N. L.: *Biochem. J.*, **30**, 1862-1869 1936.
8. FRITZ, I. B.: *Physiol. Rev.*, **41**, 52-129, 1961.
9. HALPERIN, M. L., ROBINSON, B. H. and FRITZ, I. B.: *Proc. Nat. Acad. Sci. USA*, **69**, 1003-1007, 1972.
10. KREBS, H. A.: *Advances in Enzyme Regulation* (WEBER, G., ed.), vol. 4, Pergamon Press, Oxford, 1966, pp. 339-353.
11. KREBS, H. A. and HENSELEIT, K.: *Hoppe-Seyl. Z.*, **210**, 33-66, 1932.
12. KORNBERG, A. and PRICER, W. E. Jr.: *J. Biol. Chem.*, **204**, 345-357, 1953.
13. LOFFLER, G., MATSCHINSKY, F. and WEILAND, O.: *Biochem. Z.*, **342**, 76-84, 1965.
14. MCGARRY, J., MANNAERTS, P. and FOSTER, D. W.: *J. Clin. Invest.*, **60**, 265-270, 1977.
15. MCGARRY, J., LEATHERMAN, F. and FOSTER, D. W.: *J. Biol. Chem.*, **253**, 4129-4136, 1978.
16. MCGARRY, J., TAKABAYASHI, Y. and FOSTER, D. W.: *J. Biol. Chem.*, **253**, 8294-8300, 1978.
17. MICHAL, G. and BERMEYER, H. W.: In «Methods of Enzymatic Analysis» (BERMEYER, H. H., ed.), vol. 4, Academic Press, New York, 1974, pp. 1975-1981.
18. SHEPHERD, D., YATES, D. W. and GARLAND, P. B.: *Biochem. J.*, **98**, 3c, 1966.
19. WEILAND, O.: *Adv. Metab. Disord.*, **3**, 1-47, 1968.
20. WEILAND, O. and MATCHINSKY, F.: *Life Sci.*, **1**, 49-54, 1962.
21. WEILAND, O. and LOFFLER, G.: *Boichem. Z.*, **339**, 204-211, 1963.
22. WILLIAMSON, D. H., MELLANBY, J. and KREBS, H. A.: *Biochem. J.*, **82**, 90-96, 1962.
23. WOODS, H. F., EGGLESTON, L. V. and KREBS, H. A.: *Biochem. J.*, **119**, 501-510, 1970.
24. YATES, D. W. and GARLAND, P. B.: *Biochem. Biophys. Res. Commun.*, **23**, 460-465, 1966.