PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 5-11 (1978)

Studies on the Metabolism of Glycerol by Hyperlipemic and Normolipemic Rats[1] (39979)

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Previously, it has been reported that the hyperlipemic, maturity-onset diabetic BHE rat (1) had significantly higher levels of serum free glycerol than the normolipemic, nondiabetic Wistar rat (2). The source of this glycerol was not elucidated nor was a causal relationship between the elevated glycerol and hepatic lipogenesis shown.

Recently, Lin *et al.* (3) reported that feeding large quantities of glycerol had little effect on hepatic lipogenesis as measured by the incorporation of tritium into fatty acids, while inducing an increase in the activities of citrate cleavage enzyme (EC 4.1.3.8), malic enzyme (EC 1.1.1.40), and fatty acid synthetase. If one assumes that this high dietary glycerol intake resulted in elevated serum glycerol levels, one could also assume that this experiment would produce a rat which might be similar to the BHE rat at least in so far as the relationship between serum glycerol levels and hepatic enzyme activity was concerned. One might conclude, therefore, that the elevations in serum glycerol reported to occur in the BHE rat do not have a causal relationship to the previously reported enhanced lipogenesis in the BHE rat. This hypothesis, of course, raises the question of the significance of elevated serum glycerol levels. It may well be that this "abnormality" is merely characteristic of a more fundamental error in metabolism which ultimately results in the development of hyperlipemia, maturity-onset diabetes, and vascular disease, diseases prevalent in the 300- to 400-day-old BHE rat (1, 4). For this reason we decided to investigate glycerol metabolism in the BHE rat compared to the normal Wistar rat. We restricted our inquiries to the metabolism of the liver since previous studies have shown that the major differences in lipid metabolism (as well as glucose metabolism) were observed in this organ. The study consisted of a series of experiments designed to answer the following questions in sequence: (i) Using a homogeneous BHE population, could the previously reported elevation in serum free glycerol be demonstrated? (ii) Were these differences in serum free glycerol due to (a) strain differences in heparin-induced lipoprotein lipase activity or (b) differences in the capacity of the liver to metabolize varying levels of incoming glycerol? (iii) Were there differences in the hepatic metabolism of glycerol? Using BHE and Wistar rats we found that while glycerol can enter the liver cell, its movement from the cytosol to the mitochondria is restricted, necessitating the development of alternate pathways for glycerol metabolism which, in turn, may contribute to, rather than inhibit, the lipogenic capacity of the BHE liver.

Materials and methods. Four experiments were conducted. Each experiment utilized age-matched young male BHE and Wistar[3] rats. The BHE rats used were third- and fourth-generation animals inbred[4] (brother-sister matings) to produce a homogeneous population of matu-

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[3] Wistar rats were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts.

[4] The original BHE animals were purchased from Flow Laboratories, Dublin, Virginia. The BHE colony has been moved from this facility to the NIH Animal Resource Center.

0037-9727/78/1571-0005$01.00/0

rity-onset diabetic, hyperlipemic rats. These rats differed from the rats used in previous work (1, 4) in that they were more uniform in response to carbohydrate feeding and in the development of the strain characteristics. The rats were housed in stainless-steel cages in a temperature-humidity-controlled room having equal 12-hr periods of light and dark. Food[5] and water were always available.

In the first experiment, two groups of 10 BHE and Wistar 50-day-old rats were anesthesized with sodium barbital (60 mg/kg) and blood was withdrawn by heart puncture. The serum was collected after centrifugation in the cold and used for the determination of lactate (5), pyruvate (6), β-hydroxybutyrate (7), acetoacetate (7), and glycerol (8). The second experiment utilized BHE and Wistar rats approximately 75 days old. Four groups of rats, six per group, from each strain were anesthesized with sodium barbital. Heparin (2000 units) was injected via the femoral vein, the inferior vena cava was catheterized via the jugular, and the portal vein was exposed. Saline or glycerol (35, 70, or 140 μmole/100 g body weight) was injected at 0 time into the portal vein. At 5 min prior to the injection and at 1, 3, 5, 10, 20, and 30 min after injection, blood was withdrawn from the catheter inserted into the inferior vena cava. The serum from this blood was collected after centrifugation and used for the determination of glycerol (8). Immediately after the last blood sample was drawn, the liver was quickly excised and clamped between copper plates precooled in liquid nitrogen. Perchlorate extracts of the frozen liver powders were prepared (9) and used for the determination of glycerol and α-glycerophosphate (10). Total lipid content of these powders was also determined (11).

In the third experiment, two groups of 50-day-old BHE and Wistar rats were killed by cervical dislocation, and the livers were excised and clamped between two copper plates precooled in liquid nitrogen. Less than 7 sec elapsed between the start of the cervical dislocation and the clamping of the tissue. Liver powders were prepared as described above and used for the determination of glycerol, α-glycerophosphate, dihydroxyacetone phosphate (9), 3-phosphoglycerate (9), and adenine nucleotides (9). Phosphorylation states were calculated according to the equations of Veech *et al.* (12).

In the fourth experiment, two groups of 50-day-old BHE and Wistar rats (nine rats per group) were killed by decapitation and the livers excised. Mitochondria were prepared according to the procedures of Johnson and Lardy (13) and used for the determination of mitochondrial α-glycerophosphate dehydrogenase (EC 1.1.99.5) (14) and for the activity of the α-glycerophosphate shuttle.[6]

Results of the different experiments were summarized and strain differences determined using Student's *t* test. Differences with a probability of less than 0.05 were considered significant.

Results. No significant differences in serum lactate and pyruvate were observed (Table I). There was, however, a significant increase in β-hydroxybutyrate levels and total ketones in BHE rats compared to Wistar rats. Acetoacetate levels were significantly lower in the BHE rats than in the Wistar rats. Increases in total ketones, while at this age (50 days) not excessive (still being within the normal range), suggest that utilization of these metabolites by the peripheral tissues is not as great in the BHE rat compared to the Wistar rat. These differences suggest that BHE animals would have developed diabetes at a later age. In fact, their siblings who were maintained to the age of 300 days were indeed diabetic, with fasting glucose values ranging between 180 and 240 mg/100 ml. Normal fasting rats have glucose values of 80–90 mg/100 ml. Serum glycerol levels were higher in the BHE rats than in the Wistar rats and these findings are compatible with those reported by others (2).

[5] Purina Laboratory Animal Chow, Ralston Purina Co.

[6] Tobin, R. B., Berdanier, C. D., DeVore, V., and Ecklund, R. E., (manuscript submitted for publication).

Strain differences in the serum glycerol levels were also observed in heparin-treated animals infused with saline (Table II). These baseline values were higher than those reported in Table I, but the differences in glycerol levels can be explained as being due to the effect of heparin on lipoprotein lipase. When the values in Table I were subtracted from the initial values in Table II, it was apparent that there were no differences between the strains in the effects of heparin on lipoprotein lipase. That is, the strains did not differ in their heparin-induced lipoprotein lipase activity.

Similarly, the two strains did not differ in their clearance of the different doses of glycerol. The greater the dose of glycerol administered, the longer the time required for the glycerol level in the blood to return to its preinfusion level. At the 35- and 70-μmole dose levels, less than 30 min were required for the animals to return to their preinfusion glycerol level. At the 140-μmole level, however, it appeared that more than 30 min would be required before the animals would return to their preinfusion level. These observations suggest that the high serum glycerol levels reported in Table I were not due to a decreased hepatic clearance of glycerol by BHE rats. In addition, the lack of strain differences in the clearance of glycerol from the blood indicates that in the young rat (75 days) the liver cell can rapidly adapt to changes in the concentration of this substrate and metabolize it rapidly.

This suggestion is supported by the lack of strain difference in the levels of glycerol in the liver tissue frozen immediately after the last blood sample was drawn. Serum glycerol levels were higher in the BHE rats infused with either 35 or 70 μmoles of glycerol/100 g body weight, but these differences were apparent even before the infusion began, whereas the animals infused with the highest glycerol levels had not yet recovered from this treatment. When the ratios of serum to liver glycerol levels were calculated (Table III), BHE rats had higher ratios than Wistar rats when they were infused with saline or 70 or 140 μmoles of glycerol. At the lowest glycerol dose, however, there were no strain differences in this ratio, although at this dose the ratios for BHE rats were much lower than for rats of the other treatment groups. Ratios higher than 1 indicate that more glycerol was found in the serum than in the liver tissue. Ratios less than 1 indicate that the glycerol in the serum had been rapidly transferred into the liver cell and metabolized. The glycerol could have been phosphorylated to α-glycerophosphate via glycerol kinase

TABLE I. BLOOD METABOLITES IN NONFASTED BHE AND WISTAR RATS.

Metabolites (m*M*)	Strain	
	BHE	Wistar
Lactate	3.06 ± 0.29[a]	3.59 ± 0.23
Pyruvate	0.009 ± 0.005	0.090 ± 0.005
β-Hydroxybutyrate	0.298 ± 0.037	0.213 ± 0.013*
Acetoacetate	0.019 ± 0.002	0.025 ± 0.002*
Glycerol	0.089 ± 0.012	0.050 ± 0.005*

[a] Mean ± SEM for 10 rats.
* Strain differences are significant ($P < 0.05$).

TABLE II. EFFECT OF VARYING INTRAVENOUS GLYCEROL INJECTIONS ON THE TRANSHEPATIC BLOOD GLYCEROL LEVELS

Dose (μmoles/100 g)	Minutes after glycerol						
	−5	1	3	5	10	20	30
Wistar							
0	0.164 ± 0.034[a]	0.095 ± 0.032	0.072 ± 0.014	0.112 ± 0.014	0.126 ± 0.020	0.112 ± 0.028	0.121 ± 0.022
35	0.094 ± 0.030	1.000 ± 0.080	0.25 ± 0.048	0.155 ± 0.031	0.158 ± 0.004	0.102 ± 0.005	0.104 ± 0.007
70	0.202 ± 0.007	3.170 ± 0.700	0.369 ± 0.009	0.221 ± 0.006	0.183 ± 0.002	0.180 ± 0.004	0.207 ± 0.003
140	0.184 ± 0.003	7.280 ± 2.210	3.547 ± 0.395	1.500 ± 0.590	0.721 ± 0.300	0.465 ± 0.143	0.281 ± 0.009
BHE							
0	0.244 ± 0.008*	0.144 ± 0.017	0.133 ± 0.010*	0.159 ± 0.16*	0.167 ± 0.015*	0.227 ± 0.031*	0.230 ± 0.034*
35	0.216 ± 0.090*	1.330 ± 0.220	0.279 ± 0.050	0.218 ± 0.003*	0.193 ± 0.002*	0.196 ± 0.002*	0.180 ± 0.044*
70	0.221 ± 0.064*	2.470 ± 0.620	0.910 ± 0.265	0.641 ± 0.249	0.293 ± 0.012*	0.237 ± 0.036*	0.428 ± 0.017*
140	0.235 ± 0.006*	5.740 ± 1.600	3.230 ± 0.440	1.950 ± 0.310	1.420 ± 0.630	0.378 ± 0.070	0.293 ± 0.008

[a] Mean micromoles per milliliter ± SEM for six rats.
* Strain differences are significant ($P < 0.05$).

TABLE III. SERUM GLYCEROL, LIVER GLYCEROL, AND α-GLYCEROPHOSPHATE LEVELS 30 min AFTER THE INJECTION OF VARYING LOADS OF GLYCEROL.

Dose of glycerol (μmoles/ 100 g body wt)	Serum glycerol (μmoles/ml)	Liver glycerol (μmoles/g)	Serum/liver glycerol	Liver α-glycero-phosphate (μmoles/g)	α-glycerophos-phate/glycerol
Wistar					
0	0.121 ± 0.022[a]	0.14 ± 0.04	0.85 ± 0.05	0.93 ± 0.28	6.57 ± 0.07
35	0.104 ± 0.007	0.21 ± 0.03	0.48 ± 0.04	1.24 ± 0.24	5.90 ± 0.08
70	0.207 ± 0.003	0.51 ± 0.31	0.41 ± 0.01	1.69 ± 0.85	3.31 ± 0.03
140	0.281 ± 0.009	0.24 ± 0.06	1.17 ± 0.06	1.10 ± 0.42	4.58 ± 0.08
BHE					
0	0.230 ± 0.034*	0.22 ± 0.03	1.05 ± 0.01*	1.10 ± 0.82	5.00 ± 0.04*
35	0.180 ± 0.044*	0.42 ± 0.20	0.44 ± 0.05	1.21 ± 0.60	2.88 ± 0.03*
70	0.428 ± 0.017*	0.34 ± 0.06	1.16 ± 0.03*	1.44 ± 0.71	3.88 ± 0.10*
140	0.293 ± 0.008	0.25 ± 0.05	1.27 ± 0.02*	0.97 ± 0.56	4.24 ± 0.07*

[a] Mean ± SEM for six rats.
* Strain differences are significant ($P < 0.05$).

or dehydrogenated to form glyceraldehyde through the action of aldose reductase. Usually, glycerol is phosphorylated rather than dehydrogenated since the K_m of glycerol kinase for glycerol is lower than the K_m of the aldose reductase for this metabolite (15). Glycerol kinase has been reported to be more active in BHE rats than in Wistar rats (2), but if this were true then one would expect BHE rats to have higher ratios of α-glycerophosphate to glycerol than Wistar rats. No strain differences in the amount of α-glycerophosphate were found, but when ratios of this metabolite to glycerol were calculated BHE rats had lower ratios, again suggesting that these rats probably did not have more glycerol kinase activity than Wistar rats. The lack of agreement between these findings and those reported by Gardner and Reiser (2) may have been due to the fact that an *in vitro* assay such as that used by Gardner and Reiser provides all the necessary substrates and cofactors in optimal amounts, amounts which may not be present *in vivo*. Thus, an increase in an *in vitro* enzyme activity may be an artifact of the measuring system. No strain differences were observed in the liver lipid content of the tissue powders. BHE livers contained 33.9 ± 8.5 mg of lipid/g of liver and Wistar livers contained 35.3 ± 8.5 mg of lipid/g of liver. Treatment differences were negligible. These observations are consistent with previous reports in that the strain differences in liver lipid do not appear until the animal is mature (more than 100 days old) (1, 4).

In liver tissue from rats not subjected to glycerol infusion, heparin, or anesthesia, no strain differences in hepatic glycerol, α-glycerophosphate, dihydroxyacetone phosphate, or 3-phosphoglycerate were observed (Table IV). The strain differences in the ratio of α-glycerophosphate to glycerol were again observed. BHE rats had lower ratios than Wistar rats, indicating a less active phosphorylation of glycerol to α-glycerophosphate. At the same time, these rats had higher phosphorylation states than Wistar rats. The higher phosphorylation states were due to an increase in ATP and decreases in ADP and AMP in BHE rats compared to Wistar rats.

While no differences in the endogenous α-glycerophosphate shuttle activities were observed, significant strain differences in the activities of the complete shuttle were observed (Table V). BHE isolated mitochondria had less shuttle activity in both state 3 and state 4 conditions (with or without added ADP). While significantly greater cytosolic α-glycerophosphate dehydrogenase activity has been reported (1, 4) in BHE rats, the activity of the corresponding enzyme in the mitochondria was significantly less in this strain than in the Wistar strain (Table VI). These findings are compatible with the findings of decreased mitochondrial α-glycerophosphate shuttle activity in BHE rats.

Discussion. While the results of the

present study did not identify the source of the "extra" glycerol in the serum, they did demonstrate that the BHE liver, like the Wistar liver, was capable of disposing of large loads of free glycerol. The normal half-life of this metabolite has been reported to be approximately 11 min (16). Our studies confirm these findings; in the 35- and 70-μmole groups the serum glycerol levels returned to their preinfusion levels within 20 min, with no significant changes in liver glycerol levels 30 min after the glycerol load.

From the results it is also clear that increasing the level of incoming glycerol to the liver did not increase the liver lipid content; findings which agree, in part, with those of Lin *et al.* (3). It has been reported that 400-day-old BHE rats had large quantities of hepatic glycerol (17). Apparently, in the BHE rat, glycerol accumulates in the liver because the means for its disposal are not fully active. This becomes apparent as the animal ages. Some evidence for this less active glycerol metabolism is seen in the strain differences in the ratios of serum to liver glycerol and liver α-glycerophosphate to glycerol (Tables III and IV). BHE rats had lower ratios of these liver metabolites than Wistar animals, indicating that in these rats the glycerokinase reaction was rate limiting. *In vitro* the enzyme has been shown to be more active in BHE rats than in Wistar rats (2). However, *in vitro* assays do not always replicate *in vivo* conditions and since the conversion of glycerol to α-glycerophosphate was less, one must assume that some regulatory factors are active *in vivo* and result in a less active enzyme. It has been reported that glycerol kinase activity is dependent on adequate levels of ATP (15, 18). Inspection of the data presented in Table IV shows that the BHE livers had greater quantities of ATP than Wistar livers. Similarly, glycerol ki-

TABLE IV. LIVER METABOLITES AND PHOSPHORYLATION STATES IN NONFASTED BHE AND WISTAR RATS.

	Strain (μmoles/g of liver)	
	BHE	Wistar
Glycerol	46.4 ± 3.9[a]	45.2 ± 9.1
α-Glycerophosphate	214 ± 52	260 ± 22
α-Glycerophosphate/glycerol	4.86 ± 0.28	7.13 ± 1.27*
Dihydroxyacetone phosphate	50.1 ± 5.3	47.4 ± 2.8
3-Phosphoglycerate	476 ± 32	406 ± 43
ATP	2551 ± 80	2270 ± 50*
ADP	1136 ± 45	1346 ± 26*
AMP	246 ± 29	402 ± 19*
P_i	4691 ± 412	4968 ± 465
(ATP)/(ADP) (HPO_4^{2-}) cytosol	838 ± 80	596 ± 50*
(ATP)/(ADP) (HPO_4^{2-})[b] mito $\times 10^{-2}$	3.47 ± 0.53	2.10 ± 0.25*

[a] Mean ± SEM of eight rats.
[b] Calculated from the values of the compartmented metabolites and redox state of each group (24).
* Strain differences are significant ($P < 0.05$).

TABLE V. α-GLYCEROPHOSPHATE SHUTTLE ACTIVITY IN INTACT LIVER MITOCHONDRIA FROM BHE AND WISTAR RATS.

	Micromoles of NAD produced per milligram of protein per minute			
	Endogenous[a]		Complete[b]	
Strain	State 4 (−ADP)	State 3 (+ADP)[c]	State 4 (−ADP)	State 3 (+ADP)
BHE	1.24 ± 0.68 (8)[d]	0.84 ± 0.36 (8)	6.52 ± 0.58 (9)	5.14 ± 0.46 (9)*
Wistar	1.38 ± 0.58 (8)	0.09 ± 0.35 (8)	12.64 ± 1.50 (9)**	9.96 ± 0.80 (9)*, **

[a] Mitochondrial protein, 5 mg, was suspended in a medium containing 75 m*M* mannitol, 10 m*M* phosphate buffer (pH 7.4), 75 m*M* KCl, 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 7.4), 5 m*M* NADH, and water to a volume of 3.0 ml. Flasks were incubated for 20 min at 37°.
[b] The complete system contained in addition to the above, 20 m*M* DL-α-glycerophosphate, 5 units of α-glycerophosphate dehydrogenase, and 30 nmoles of rotenone/g of protein to a volume of 3 ml.
[c] ADP, 4 m*M*, was added where indicated.
[d] Mean ± SEM; number of samples in parentheses.
* Differences in NAD production due to the addition of ADP significant ($P < 0.05$).
** Strain differences in NAD production are significant ($P < 0.001$).

TABLE VI. MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY.

Strain	Enzyme activity (μmoles of O_2/mg of mitochondrial protein/min)
BHE	9.2 ± 1.1[a]
Wistar	12.0 ± 1.1*

[a] Mean ± SEM of eight rats per group.
* Strain differences are significant ($P < 0.05$).

nase can be inhibited by ADP (15); again, the data in Table IV would suggest that the enzyme is not so inhibited. Some inhibition of the enzyme might be anticipated if there were some strain differences in the distribution and exchange of adenine nucleotides. This does not appear likely though, in view of the fact that the ATP/ADP · HPO_4 ratios in both the cytosol and mitochondria are greater in BHE rats than in Wistar rats. If there were differences in adenine nucleotide exchange, then one might anticipate a discordance in the phosphorylation states of the two compartments. One cannot rule this out, however, until measures of adenine nucleotide translocase are made.

The liver cell actively transports glycerol into the cell (16, 18) to the extent that an equilibrium between the intracellular and extracellular compartments is seldom established. As glycerol is rapidly metabolized within the cell, more extracellular glycerol diffuses in. In Wistar rats, glycerol does not accumulate in either the serum or the liver and therefore one must assume that the accumulation seen in the BHE rat is due to some constraint in glycerol metabolism within the cell. Conceivably this constraint could include a less active glycerol kinase and/or a less active mitochondrial shuttle system.

Houstek *et al.* (20) have suggested that the α-glycerophosphate shuttle serves a regulatory function in the metabolism of α-glycerophosphate by brown adipose tissue. In liver, it may also serve to regulate metabolism by suppressing palmitylcarnitine conversion to palmityl CoA (21). When mitochondrial α-glycerophosphate dehydrogenase is low, the activity of the shuttle is low and β oxidation of fatty acids is not suppressed. This has a net effect of increasing the mitochondrial NAD^+/NADH ratio as well as increasing the ATP/ADP ratio (21–23). Changes in the oxidation of palmitylcarnitine can affect the relative distribution of the adenine nucleotides within the cell through an inhibitory effect of acyl CoA on ATP translocase (23, 24). Any disruption of this transport process would be reflected in a lack of a coordination between the metabolic processes of the intra- and extramitochondrial compartments of the cell (23). While further work must be conducted in order to firmly establish in the BHE rat that such changes in metabolic regulation have occurred, the present data can be taken as strong suggestive evidence of such changes. Lower α-glycerophosphate shuttle activity has been shown (Table V), as have lower mitochondrial α-glycerophosphate dehydrogenase activity and higher phosphorylation states (Tables VI and IV). All of these observations are compatible with the previously reported decrease in energy utilization versus energy stored as fat (4).

Summary. The metabolism of glycerol has been studied in BHE and Wistar rats using measures of transhepatic glycerol disposal, assays of glycerol and glycerol metabolites in frozen clamped hepatic tissue, and through the study of mitochondrial α-glycerophosphate shuttle activity and α-glycerophosphate dehydrogenase activity. Strain differences in glycerol metabolism were observed, with the BHE rats having higher serum glycerol levels, lower presumed glycerolkinase activity, lower α-glycerophosphate shuttle activity, and lower mitochondrial α-glycerophosphate dehydrogenase activity. These findings were presumed to indicate a lack of coordination between the cellular compartments, explaining the previously reported enhanced hepatic lipogenesis seen in BHE rats.

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Received July 15, 1977. P.S.E.B.M. 1978, Vol. 157.