Hepatic Mitochondrial Function in Ketogenic States

DIABETES, STARVATION, AND AFTER GROWTH HORMONE ADMINISTRATION

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ABSTRACT The study was designed to evaluate hepatic mitochondrial function during ketotic states. The ketogenic models studied were streptozotocin-induced diabetic ketoacidosis, 48 h of starvation, and after growth hormone administration. In the last-mentioned model we observed increased free fatty acids but not ketonemia.

Oxidative phosphorylation was measured using the citric acid cycle substrates pyruvate and succinate, the amino acid glutamate, a ketone body β-hydroxybutyrate, and a long-chain fatty acid palmitoyl-*l*-carnitine. State 3 (ADP stimulated) and state 4 (ADP limited) respiration, respiratory control ratio (state 3/state 4), and the ADP/O ratios were normal in the controls and the experimental groups. Uncoupled respiration produced by dinitrophenol with a variety of substrates was unchanged in the experimental groups compared to the controls.

Fatty acid oxidation was studied in detail. The rate of utilization of palmitoyl-*l*-carnitine by controls or experimental groups did not depend on the product formed (citrate, acetoacetate). No significant changes were observed in the oxidation of palmitoyl-CoA (+ carnitine) or with an intermediate-chain fatty acid hexanoate. The specific activity of hepatic mitochondria carnitine palmitoyltransferase did not change in any of the three experimental groups.

It is concluded that during diabetic ketoacidosis, starvation, and growth hormone administration, there is (*a*) no alteration in hepatic mitochondrial function; (*b*) no change in the intrinsic capacity of hepatic mitochondria to oxidize fatty acids; and (*c*) no change in the specific activity of mitochondrial carnitine palmitoyltransferase. The mechanism by which the body restrains flux through the mitochondrial oxidative machinery remains to be fully determined.

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INTRODUCTION

Ketosis is a physiological response to a diverse group of hormonal or nutritional perturbations. Ketotic states are characterized by increased rates of both hepatic ketogenesis and extrahepatic ketone body utilization (1, 2). Diabetes mellitus, starvation, and growth hormone excess have served as models for investigating the regulation of ketosis. Perfused livers isolated from either starved or diabetic animals oxidize fatty acids to ketone bodies at faster rates than do livers from control animals under the same perfusion conditions (3–6). During ketosis, plasma free fatty acid (FFA) concentrations are elevated, but studies on perfused livers have shown that increased substrate availability alone cannot explain the observed differences in ketone body production and that some other intrahepatic factors must be contributing (3). Many in vivo and in vitro systems have been used to investigate possible mechanisms for the regulation of ketogenesis, but the physiological factors responsible for increased rates of fatty acid oxidation to ketone bodies are not fully understood.

Since hepatic mitochondria are the major sites of ketone body formation, considerable attention has been focused on the factors controlling mitochondrial function. Partial uncoupling of mitochondrial respiration has been used to explain the alterations in lipid metabolism during diabetic ketoacidosis (7–9) but these findings have been questioned (10, 11). Regulatory phenomena at several of the steps involved in fatty acid oxidation have been proposed. The mitochondrial sites implicated have included the transport of acyl

groups (12), the β-oxidation cycle (13), and product formation, (citrate and acetoacetate) (14). Recently, attention has been given to intrinsic changes in mitochondrial enzyme activity as a regulatory factor in ketosis (13, 15–17). Increased carnitine palmitoyltransferase activity (CPT)[1] has been described in the ketosis of diabetes (15–17), starvation (15, 18), and after growth hormone administration (19). Bunyan and Greenbaum (13) have described an increase in acyl-CoA dehydrogenase activity in rat liver homogenates after treatment with growth hormone and used this finding to explain an observed increase in fatty acid oxidation.

Although both in vivo and in vitro studies have demonstrated increased rates of fatty acid oxidation and ketone body formation in the three conditions described above, the role of the mitochondrion itself in the process is controversial. We have measured oxidative phosphorylation in hepatic mitochondria isolated from normal, diabetic, starved, and growth hormone-treated rats. In addition, the rates of hepatic oxidation of fatty acids have been calculated and the specific activity of CPT in the hepatic mitochondria determined.

METHODS

Animals. All animals were housed in large, wire-bottomed cages with free access to laboratory chow and water. Diabetes was induced in male Sprague-Dawley strain rats (280–400 g) with a single injection of streptozotocin (85 mg/kg). The drug was dissolved in 50 mM citrate buffer, pH 4.0 and immediately injected into the tail vein of the animals under light ether anesthesia. Controls received an injection of the buffer only. All the rats given streptozotocin showed 3+ glycosuria and 1+ ketonuria (Lab-stix, Ames Co., Div., Miles Labs., Inc., Elkhart, Ind.) within 24 h. The diabetic animals were then maintained on a daily dose of 2.5 U of protamine zinc insulin (Eli Lilly and Company, Indianapolis, Ind.) subcutaneously for 8–10 days, as suggested by Wieland (1). They were sacrificed 96 h after their last dose of insulin.

Adult, male Wistar strain rats were used for the experiments on starvation and growth hormone administration. The starved rats and their controls initially weighed between 150–200 g. Animals were starved by removing all food from their cages 48 h before sacrifice. Bovine growth hormone (bGH) was administered by subcutaneous injection after suspension of the crystalline hormone preparation in 0.9% NaCl adjusted to pH 9.0 with NaOH. The rats received either two injections of 1 mg of bGH at 24 and 4 h before sacrifice or two injections of saline at the same times. Both control and experimental animals were kept without food for the 4-h period between the last injection and sacrifice.

Mitochondrial isolation. When killed, the animals were stunned with a blow to the head and decapitated. Blood was collected into chilled beakers containing a few drops of a 1:1,000 heparin solution. The liver was removed, rinsed, blotted, and weighed. All subsequent procedures were carried out at 0°C. A 10% tissue homogenate was prepared with a Potter-Elvehjem homogenizer in a medium containing 220 mM mannitol, 70 mM sucrose, 5 mM morpholinopropane sulfonic acid (MOPS), 2 mM EDTA (pH 7.4). After removal of nuclei and cell debris by centrifugation at 700 *g* for 10 min, mitochondria were isolated by centrifugation at 7,000 *g* for 10 min and washed twice. The final pellet was resuspended to a protein concentration of 50 mg/ml in 220 mM mannitol, 70 mM sucrose, and 5 mM MOPS (pH 7.4). Protein was measured by the biuret reaction (20).

Oxygen electrode. Oxygen consumption experiments were carried out using a Clark-type oxygen electrode in a 1-ml water-jacketed chamber at 30°C. The incubation medium contained 80 mM KCl, 50 mM MOPS, 1 mM ethylene glycol bis(β-aminoethyl ester)-*N,N,N',N'*-tetraacetic acid (EGTA), and 5 mM P_i at pH 7.0. During measurement of oxidative phosphorylation, defatted, dialyzed bovine serum albumin was included in the medium at a concentration of 1 mg/ml. The designations state 3 for ADP-stimulated respiration and state 4 for ADP-limited respiration are those used by Chance and Williams (21). The methods for calculating the respiratory control ratios (RCR) and the ADP/O ratio have been described by Estabrook (22). The ratio of oxygen consumed to palmitoyl group utilized ($\Delta O/\Delta P$) was determined as described by Garland, Shepherd, Nicholls, Yates, and Light (14). The procedure involved depleting mitochondria; of endogenous substrates (in the incubation medium) a known amount (8.6 nmol) of palmitoyl-*l*-carnitine was then added and the amount of oxygen consumed due to this substrate measured. The amount of oxygen consumed is dependent on the product formed (citrate, acetoacetate, or CO_2) from the oxidation of palmitoylcarnitine and can be used as an indirect measurement of the product formed (14). The rate of substrate (palmitoyl group) utilization was measured from the time required to oxidize the added palmitoylcarnitine; this rate can also be calculated by dividing the rates of oxygen consumption by the $\Delta O/\Delta P$.

Assay of CPT activity. CPT activity was measured with the CPT-assay 2 as previously described (23). The assay measures the liberation of radioactive carnitine from palmitoyl-*l*-[*methyl*-^{14}C]carnitine in the presence of 4 mM CoA and 5 μg of mitochondrial protein. The activity is expressed as nanomoles radioactive carnitine liberated per minute per milligram mitochondrial protein. Mitochondrial preparations were stored at −80°C before these assays were performed.

Materials. Bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) was defatted by the method of Chen (24) and dialyzed as described by Hanson and Ballard (25). Palmitoyl-*l*-carnitine was synthesized by the method of Ziegler, Bruckner, and Binon (26). *l*-[*methyl*-^{14}C]Carnitine was synthesized by the method of Goodfellow and Hoppel (unpublished observations). Palmitoyl-*l*-[*methyl*-^{14}C]carnitine was synthesized from *l*-[*methyl*-^{14}C]carnitine and palmitoyl chloride by the method of Brendel and Bressler (27). The concentration of palmitoylcarnitine in solution was determined by the method of Hestrin (28) modified to contain 50% methanol. Palmitoyl-CoA was prepared as described by Seubert (29). Streptozotocin (U-9889, lot no. 9861-GGS-118PI) was a gift from Dr. W. E. Dulin of The Upjohn Company (Kalamazoo, Mich.). bGH was a gift of the National Institute for Arthritis and Metabolic Disease. The preparation (NIH-GH-B17) had an esti-

[1] *Abbreviations used in this paper:* bGH, bovine growth hormone; CPT, carnitine palmitoyltransferase; DNP, dinitrophenol; MOPS, morpholinopropane sulfonic acid; RCR, respiratory control ratios.

TABLE I

Experimental Parameters in Diabetes, Starvation, and after Growth Hormone Administration

Condition	Parameter	Control*	Experimental*
Starvation‡	Weight change in 48 h, *g*	+9.8±1.8	−42.3±7.9
Diabetes§	Weight change in 96 h, *g*	—	−35.4±8.3
	Plasma glucose, *mg/100 ml*	140.4±18.5	527.1±79.8
	Plasma FFA, *meq/liter*	0.16±0.30	1.00±0.41
	Plasma triglycerides, *mg/100 ml*	88.3±18.5	864±448
Growth hormone‖	Weight change in 48 h, *g*	−1.25±3.25	+0.5±3.87
	Plasma FFA, *meq/liter*	0.34±0.09	0.65±0.06
	Plasma glucose, *mg/100 ml*	127.4±9.8	135.3±3.9

* Values are reported as mean ±SD for four to five animals in each group.

‡ Rats were starved for 48 h before sacrifice.

§ Streptozotocin-induced diabetic rats were maintained on protamine zinc insulin (2.5 U/day) for 8–10 days. Insulin was then stopped and the rats sacrificed 96 h later. Controls received buffer only.

‖ Rats received 1 mg bGH subcutaneously 24 h and again 4 h before sacrifice and food was removed for the 4-h period before sacrifice. Controls received saline.

mated potency of 0.92 IU/mg and was essentially free of contamination with thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, or prolactin. *l*-Carnitine chloride was a gift from the Otsuka Pharmaceutical Co. (Osaka, Japan). CoA, ATP, and ADP were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. All other chemicals and reagents were of the highest available purity. ADP solutions were assayed by the method of Chappell (30). Plasma concentrations of glucose (31), FFA (32), and triglycerides (33) were measured using standard techniques. Blood β-hydroxybutyrate and acetoacetate were determined by the fluorimetric enzyme method of Olsen (34).

RESULTS

Experimental parameters (Table I). Animals starved for 48 h lost approximately 15% of their body weight. Blood β-hydroxybutyrate (0.330±0.110 mM) and acetoacetate (0.080±0.006 mM) were significantly elevated over the controls (β-hydroxybutyrate = 0.029± 0.002 mM; acetoacetate = 0.015±0.012 mM). Diabetic animals lost weight after receiving streptozotocin but regained their weight rapidly and continued to gain throughout the period of insulin replacement. In the 4 days after insulin withdrawal the animals underwent a 10–15% weight loss. The diabetic animals showed marked elevations of plasma glucose, FFA, and triglycerides. Plasmas from four out of the five diabetic animals were grossly lipemic and the turbidity did not float at the surface after standing at 4°C overnight, indicating that the triglycerides were contained in a very low density lipoprotein fraction. Rats given growth hormone at 24 and 4 h before killing and fasted for 4 h after the second injection showed a moderate elevation of plasma FFA over controls ($P < 0.05$) but no significant elevation in blood ketone bodies. Without the 4-h period of fasting after the second injection of growth hormone, changes were not seen in the levels of plasma FFA. The growth hormone model was used even though we did not observe significant ketonemia.

Oxidative phosphorylation. The hepatic mitochondria isolated from starved or diabetic rats show normal rates of state 3 and state 4 respiration, RCR, and ADP/O ratios (Table II) for all substrates tested. Substrates studied included the citric acid cycle intermediate succinate (plus rotenone to inhibit reversed electron transport); an amino acid glutamate, and a ketone body β-hydroxybutyrate; both of which have their own specific mitochondrial dehydrogenases; and pyruvate which, like the fatty acids (palmitoylcarnitine and palmitoyl-CoA), is a source of acetyl-CoA for either citrate or acetoacetate synthesis.

Uncoupled respiration. Respiration in hepatic mitochondria from starved, diabetic, or rats treated with bGH was uncoupled by the addition of dinitrophenol (DNP) (Table III). The rates of oxidation were normal for the citric acid cycle substrates α-ketoglutarate and succinate (plus rotenone), the amino acid glutamate, and both an intermediate-chain fatty acid hexanoate and the long-chain acyl-CoA palmitoyl-CoA (plus carnitine).

Palmitoylcarnitine oxidation. Oxygen consumption during the oxidation of limited amounts of palmitoylcarnitine was monitored during respirations under three different conditions: In the presence of malate, where citrate is the chief product ($\Delta O/\Delta P = 22$); in the presence of malonate, where acetoacetate is formed ($\Delta O/\Delta P = 14$); and in the presence of oxalacetate and rotenone, where oxygen consumption is only due to the dehydrogenations of saturated acyl-CoA during β-oxidation ($\Delta O/\Delta P = 7$) (35). As shown in Table IV, mitochondria

TABLE II

*Oxidative Phosphorylation in Hepatic Mitochondria from Starved or Diabetic Rats**

Substrate(s)	Group‡	Oxygen consumption		RCR§	ADP/O
		State 3	State 4		
		natoms O·min^{-1}·mg^{-1}			
40 μM Palmitoylcarnitine	Control	89.0±6.3	6.7±4.9	13.3	2.38±0.04
+2.5 mM L-malate	Starved	87.2±7.5	8.3±3.0	10.5	2.36±0.02
	Control	72.1±14.9	9.1±4.8	8.0	2.48±0.32
	Diabetic	73.3±12.7	9.1±4.9	8.1	2.62±0.09
40 μM Palmitoyl-CoA					
+2 mM *l*-Carnitine	Control	70.2±5.7	7.5±4.0	9.4	2.69±0.05
+2.5 mM L-Malate	Starved	71.5±7.0	7.6±4.5	9.4	2.70±0.13
10 mM Pyruvate	Control	38.7±10.8	6.9±5.2	5.6	2.99±0.06
+2.5 mM L-Malate	Starved	34.1±4.9	6.1±7.7	5.7	2.97±0.56
	Control	41.4±10.5	6.3±5.3	6.6	3.50±0.69
	Diabetic	40.9±7.8	5.4±3.0	7.6	3.37±0.23
20 mM D,L-β-Hydroxybutyrate	Control	46.7±4.5	1.2±0	38.9‖	3.62±0.11
	Starved	41.1±5.2	1.7±1.0	24.1‖	3.73±0.39
	Control	49.6±5.8	7.5±5.6	6.7	2.91±0.33
	Diabetic	49.5±8.3	7.1±4.7	7.0	3.21±0.52
10 mM Glutamate	Control	68.3±4.8	5.4±5.2	12.1	2.99±0.50
	Diabetic	67.5±4.8	8.6±5.3	7.9	2.85±0.30
10 mM Succinate	Control	133.4±13.3	16.6±4.9	8.0	1.94±0.20
+3.75 μM Rotenone	Starved	120.8±11.4	13.6±8.7	8.9	1.83±0.12
	Control	101.3±15.5	16.5±4.1	6.1	1.73±0.04
	Diabetic	102.9±19.8	18.9±5.5	5.5	1.63±0.12

* Data are presented as mean ±SD. The means are not significantly different.

‡ Groups were comprised of hepatic mitochondrial preparations from four control, four starved, or five diabetic rats.

§ RCR, respiratory control ratio (mean state 3/mean state 4).

‖ RCRs above 20 were not considered to be significantly different from 20.

from starved, diabetic, or bGH-treated rats did not show any changes in their ability to oxidize palmitoylcarnitine regardless of the product formed. The calculated rates of palmitoyl group utilization were relatively constant whether either citrate or acetoacetate was the metabolic product.

CPT activity. Table V shows the specific activity of CPT measured in each of the control and experimental groups. The specific activity of CPT far exceeds the maximal rates of palmitoyl group utilization in intact mitochondria. When expressed on the basis of mitochondrial protein, the CPT activity does not change during diabetes, starvation, or after bGH treatment. Since CPT activity is exclusively mitochondrial (23), we calculated the activities per gram wet weight liver based on the observed yield of mitochondrial protein. The CPT activity per gram wet weight of liver increases in starvation or decompensated diabetes in parallel to the increase in mitochondrial protein yield (the yield in milligram mitochondrial protein per gram wet weight liver ±SD was 25.1±1.9 in the starved rats compared to 15.2±1.4 in the controls and in diabetic animals it was 19.1±2.5 compared to 15.3±2.5 in the controls). No significant change was observed in CPT activity in bGH-treated rats. When CPT activity is expressed per animals, no differences are observed between the control and experimental groups. This reflects the change in liver weight which was 6.0±0.1 g per 257.8±18.7 g initial body weight in starved rats compared to 10.9± 1.0 g liver per 256.0±22.0 g initial body weight of the controls and for diabetic rats the liver weight was 11.0± 0.7 per 347.6±30.8 g at insulin withdrawal compared

TABLE III

*Uncoupled Respiration in Hepatic Mitochondria**

Substrate(s)	Oxygen uptake					
	Control	Starved	Control	Diabetic	Control	bGH
	natoms O·min⁻¹·mg⁻¹					
0.017 mM Palmitoyl-CoA +2 mM *l*-Carnitine +2.5 mM L-Malate	75.4±7.3	87.4±11.9	85.3±8.4	93.8±4.9	73.8±9.6	76.3±9.1
0.4 mM Hexanoate‡ +2.5 mM L-Malate +1 mM ATP +2.5 μg Oligomycin/ml	66.2±11.0	59.8±7.1	59.2±3.6	55.2±10.6	71.1±13.8	72.0±8.9
10 mM L-Glutamate	48.9±2.3	56.9±5.9	50.3±3.3	57.9±9.1	—	—
10 mM α-Ketoglutarate +10 mM Malonate	—	—	19.0±5.0	22.3±3.7	21.2±4.5	19.7±2.8
10 mM Succinate +3.75 μM Rotenone	118.9±8.1	127.5±8.6	—	—	104.9±16.6	102.5±11.7

* 0.1 mM DNP was used to produce uncoupled respiration. Data are presented mean ±SD. The means are not significantly different.

‡ Hexanoate oxidation is dependent on intramitochondrial ATP; oligomycin is added to inhibit the DNP stimulation of ATPase.

TABLE IV

*Fatty Acid Oxidation by Hepatic Mitochondria**

		Oxygen consumption	ΔO/ΔP‡	Palmitoyl group utilization
		natoms O·min⁻¹·mg⁻¹		*nmol·min⁻¹·mg⁻¹*
0.0086 mM Palmitoyl-*l*-Carnitine +2.5 mM L-Malate +0.1 mM DNP	Control	88.7±12.3	22.7±1.0	3.91
	Starved	89.0±5.6	21.3±1.4	4.18
	Control	106.4±22.0	22.0±2.0	4.73
	Diabetic	95.8±12.2	20.8±1.1	4.61
	Control	101.3±14.8	22.8±1.4	4.45
	bGH	108.3±18.0	22.8±0.7	4.75
0.0086 mM Palmitoyl-*l*-Carnitine +5 mM Malonate +0.1 mM DNP	Control	46.2±6.4	11.5±0.6	4.03
	Starved	51.4±2.1	11.8±0.8	4.31
	Control	66.1±14.4	14.0±1.2	4.71
	Diabetic	62.1±7.0	12.3±1.2	5.05
	Control	55.8±14.0	13.3±2.9	4.19
	bGH	49.0±9.6	13.2±1.2	3.77
0.0086 mM Palmitoyl-*l*-Carnitine +10 mM Oxalacetate +3.75 μM Rotenone +2.5 mM ADP	Control	28.3±4.6	6.7±0.4	4.22
	Starved	28.1±2.0	6.8±0.8	4.11
	Control	36.0±4.0	6.8±1.1	5.26
	Diabetic	33.1±7.1	6.6±0.5	5.06
	Control	27.7±1.0	8.0±1.0	3.50
	bGH	26.1±5.2	7.4±1.2	3.56

* Data are presented as the mean ±SD. The means are not significantly different.

‡ ΔO/ΔP = nanoatoms O consumed per nanomole of palmitoyl-*l*-Carnitine.

TABLE V

*Carnitine Palmitoyltransferase in Hepatic Mitochondria**

Group	nU‡·mg^{-1} Mitochondrial protein	nU·10^{-3}·g^{-1} wet wt Liver	nU·10^{-3} Animal
Control	563.3±173.1	8.71±3.4	95.9±40.5
Starved	588.3±73.4	14.7±1.6§	89.5±15.4
Control	521.8±89.0	7.88±2.06	102.4±27.5
Diabetic	591.3±159.2	11.50±2.39‖	125.5±23.4
Control	668.0±222.0	14.3±5.9	95.8±37.2
bGH treated	586.8±171.8	10.9±1.7	80.2±11.2

* Data are presented as mean ±SD.
‡ nU = nanounit = nanomoles carnitine produced per minute.
§ $P < 0.02$ compared to control.
‖ $P < 0.1$ compared to control.

to 13.8±3.8 g liver weight per 362.8±37.3 g body weight in the controls. Again, no change was observed in CPT activity in bGH-treated rats.

DISCUSSION

The hepatic mitochondria isolated from animals with three conditions characterized by enhanced rates of fatty acid oxidation and ketone body formation in the intact animal demonstrated normal values for oxidative phosphorylation compared to mitochondria isolated from control animals. Using isolated mitochondria from diabetic and starved animals, no changes were seen in the ADP/O ratio or in the RCR during the oxidation of five different substrates. Partial uncoupling of mitochondrial respiration has been used to explain the alterations in lipid metabolism during diabetic ketoacidosis. Matsubara and Tochino (7) attributed their findings to an increased fatty acid content in the mitochondria but the results were not consistent when different ways of inducing diabetes were studied. Inclusion of defatted albumin in the incubation media as in the experiments reported here will also reverse uncoupling caused by endogenous FFA. Harano, DePalma, Lavine, and Miller (8) reported that diabetic mitochondria were partially uncoupled but this conclusion depended on only a small change (less than 6 natoms/min per mg) in the rate of state 4 oxidation. The physiological importance of a change of this magnitude in state 4 respiration is unknown. Our study showed no such change. Mackerer, Paquet, Mehlman, and Tobin (10) have also found normal oxidative phosphorylation in diabetes using α-ketoglutarate and pyruvate as substrates.

The oxidation of fatty acids was examined with a variety of conditions. First, with palmitoylcarnitine as substrate, three different incubation conditions which result in different product formation were used and oxidation rates were unchanged between the controls and each of the three experimental groups. The rate of palmitoyl group utilization was calculated and no changes were observed. The capacity of the hepatic mitochondria to use palmitoyl groups as substrates for oxidation was not dependent on the product formed or altered by the three experimental ketogenic states. Secondly, palmitoyl-CoA (+ carnitine) which requires enzymic (CPT-A or -I) transfer to palmitoylcarnitine before oxidation occurs was studied. Under conditions where citrate synthesis is favored (2.5 mM L-malate present) and in both coupled (ADP) and uncoupled (DNP) states no significant changes were seen in palmitoyl-CoA oxidation rates in any of the ketogenic states. Lastly, the oxidation of an intermediate-chain fatty acid hexanoate, was studied. This fatty acid substrate is not carnitine dependent for oxidation; instead, the inner membrane of the mitochondria is permeable to hexanoate. Hexanoate enters the β-oxidation cycle after conversion to hexanoyl-CoA in the mitochondria matrix compartment. The oxidation of hexanoate by hepatic mitochondria was unaffected in the three ketogenic states studied. Thus the capacity of hepatic mitochondria to oxidize fatty acid is not affected during starvation, diabetes, or after growth hormone administration.

Total CPT activity did not change in the three ketogenic states when expressed as specific activity per milligram mitochondrial protein or per total hepatic content. When expressed on the basis of gram wet weight liver, there was an apparent increase in activity during starvation and diabetes. This change may result from the form of the calculation. During 48 h of starvation, there is a decrease in body weight with a marked decrease in wet weight of liver and the recovery of mitochondrial protein per gram wet weight liver increases. When animals in the control group are matched for body weight, the net result after 48 h starvation is the recovery of comparable total amounts of hepatic mitochondrial protein. Thus, the activity expressed per gram wet weight of liver is reflecting the change in mitochondrial protein concentration resulting from multiple factors such as dehydration of the liver, loss of glycogen, etc. (36). In contrast to our finding, other investigators have reported increases in CPT activity during starvation (15, 18, 37) and diabetes (15). We agree that when data are expressed per gram wet weight of liver as done by Norum (15), there is an apparent increase in activity. One other possible reason for the discrepancy in data is the contribution of palmitoyl-CoA hydrolase to the assay system. Our assay (CPT-assay 2) is not influenced by this contaminating enzyme whereas the isotope-exchange reaction used by Norum (15) and Van Tol (18) may be influenced (38). We have observed that during starvation there is a de-

crease in palmitoyl-CoA hydrolase activity in the liver (Hoppel and Shapiro, unpublished observations) which would result in an increase in the isotope exchange-measured transferase activity.

Perfused livers from diabetic or starved animals show enhanced rates of fatty acid oxidation to ketone bodies, yet mitochondria isolated from liver during these conditions appear normal when their oxidative abilities are measured in vitro. The rates of palmitoylcarnitine oxidation seen in these experiments are comparable with rates obtained in experiments using perfused livers. Several factors must be considered to convert data obtained using isolated mitochondria to a form appropriate for comparison to an intact organ or animal. Normal rat liver contains approximately 25 mg of mitochondrial protein/g of tissue and the liver weight in a young adult rat is about 4.5% of total body weight. During 48 h of starvation (37) or acute diabetes (39), animals may lose 20% of their initial body weight (Table I) and 40% of their liver weight. Rates of oxidation at 30°C are approximately two-thirds that seen at 37°C. Using these factors, a rate of palmitoylcarnitine utilization of 5.5 nmol/min per mg of mitochondrial protein can be estimated to be equal to rates of 22.3 μmol of palmitate used/h per g of starved or diabetic liver or 70 μmol/h per 100 g of body weight. These values approximate the highest rates of palmitate oxidation to ketone bodies reported by Krebs, Wallace, Hems, and Freedlond (5), McGarry, Meier, and Foster (4), or Van Harken, Dixon, and Heimberg (6) in starvation and diabetes. Thus both control and experimental hepatic mitochondria under optimal in vitro conditions are capable of oxidizing palmitoyl groups to ketone bodies similar to those seen in vivo.

The mechanism by which the body restrains flux through the mitochondrial oxidative machinery remains to be fully determined. The findings that the ability of isolated hepatic mitochondria to oxidize fatty acids is large and constant imply that in vivo regulation must occur through changes other than that in mitochondrial capacity.

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REFERENCES

1. Wieland, O. 1968. Ketogenesis and its regulation. *Adv. Metab. Disord.* **3**: 1–47.
2. McGarry, J. D., and D. W. Foster. 1972. Regulation of ketogenesis and clinical aspects of the ketotic state. *Metab. (Clin. Exp.).* **21**: 471–489.
3. McGarry, J. D., and D. W. Foster. 1971. The regulation of ketogenesis from oleic acid and the influence of antiketogenic agents. *J. Biol. Chem.* **246**: 6247–6253.
4. McGarry, J. D., J. M. Meier, and D. W. Foster. 1973. The effects of starvation and refeeding on carbohydrate and lipid metabolism *in vivo* and in the perfused rat liver. The relationship between fatty acid oxidation and esterification in the regulation of ketogenesis. *J. Biol. Chem.* **248**: 270–278.
5. Krebs, H. A., P. G. Wallace, R. Hems, and R. A. Freedland. 1969. Rate of ketone-body formation in the perfused rat liver. *Biochem. J.* **112**: 595–600.
6. Van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. *J. Biol. Chem.* **244**: 2278–2285.
7. Matsubara, T., and Y. Tochino. 1969. Depression of respiratory activities in the liver mitochondria of diabetic rats and the restorative action of insulin. *J. Biochem.* **66**: 397–404.
8. Harano, Y., R. G. DePalma, L. Lavine, and M. Miller. 1972. Fatty acid oxidation, oxidative phosphorylation and ultrastructure of mitochondria in the diabetic rat liver. Hepatic factors in diabetic ketosis. *Diabetes.* **21**: 257–270.
9. Hall, J. C., L. A. Sordahl, and P. L. Stefko. 1960. The effect of insulin on oxidative phosphorylation in normal and diabetic mitochondria. *J. Biol. Chem.* **235**: 1536–1539.
10. Mackerer, C. R., R. J. Paquet, M. A. Mehlman, and R. B. Tobin. 1971. Oxidation and phosphorylation in liver mitochondria from alloxan and streptozotocin diabetic rats. *Proc. Soc. Exp. Biol. Med.* **137**: 992–995.
11. Gross, M. D., S. Harris, and R. E. Beyer. 1972. The effect of streptozotocin-induced diabetes on oxidative phosphorylation and related reactions in skeletal muscle mitochondria. *Horm. Metab. Res.* **4**: 1–7.
12. Shepherd, D., D. W. Yates, and P. B. Garland. 1966. The rate limiting step in the oxidation of palmitate or palmitoyl-Coenzyme A by rat-liver mitochondria. *Biochem. J.* **98**: 3c–4c.
13. Bunyan, P. J., and A. L. Greenbaum. 1965. The effect of treatment of rats with pituitary growth hormone on the activities of some enzymes involved in fatty acid degradation and synthesis. *Biochem. J.* **96**: 432–438.
14. Garland, P. B., D. Shepherd, D. G. Nicholls, D. W. Yates, and P. A. Light. 1969. Interactions between fatty acid oxidation and the tricarboxylic acid cycle. *In* Citric Acid Cycle. J. M. Lowenstein, editor. Marcel Dekker, New York. 163–212.
15. Norum, K. R. 1965. Activation of palmitoyl-CoA: carnitine palmityltransferase in livers from fasted, fat-fed, or diabetic rats. *Biochim. Biophys. Acta.* **98**: 652–654.
16. Harano, Y., J. Kowal, R. Yamazaki, L. Lavine, and M. Miller. 1972. Carnitine palmitoyltransferase activities (1 and 2) and the rate of palmitate oxidation in liver mitochondria from diabetic rats. *Arch. Biochem. Biophys.* **153**: 426–437.
17. McGarry, J. D., and D. W. Foster. 1973. Acute reversal of experimental diabetic ketoacidosis in the rat with (+)-decanoylcarnitine. *J. Clin. Invest.* **52**: 877–884.
18. Van Tol, A. 1974. The effect of fasting on the acylation of carnitine and glycerophosphate in rat liver subcellular fractions. *Biochim. Biophys. Acta.* **357**: 14–23.

19. J. K. Goldman, and R. Bressler. 1967. Growth hormone stimulation of fatty acid utilization by adipose tissue. *Endocrinology.* **81**: 1306–1310.
20. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum protein by means of the biuret reaction. *J. Biol. Chem.* **177**: 751–766.
21. Chance, B., and G. R. Williams. 1955. Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* **217**: 409–427.
22. Estabrook, R. W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol.* **10**: 41–47.
23. Hoppel, C. L., and R. J. Tomec. 1972. Carnitine palmitoyltransferase. Location of two enzymatic activities in rat liver mitochondria. *J. Biol. Chem.* **247**: 832–841.
24. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**: 173–181.
25. Hanson, R. W., and F. J. Ballard. 1968. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. *J. Lipid Res.* **9**: 667–668.
26. Ziegler, H. J., P. Bruckner, and F. Binon. 1967. O-acylation of *dl*-carnitine chloride. *J. Org. Chem.* **32**: 3989–3991.
27. Brendel, K., and R. Bressler. 1967. The resolution of (+)-carnitine and the synthesis of acylcarnitines. *Biochim. Biophys. Acta.* **137**: 98–106.
28. Hestrin, S. 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. Biol. Chem.* **180**: 249–261.
29. Seubert, W. 1960. S-palmitoyl CoA. *Biochem. Prep.* **9**: 80–83.
30. Chappell, J. B. 1964. The oxidation of citrate, isocitrate and *cis*-aconitate by isolated mitochondria. *Biochem. J.* **90**: 225–237.
31. Teller, J. D. 1964. Determination of blood glucose. *Chem. Abstr.* **60**: 7136f.
32. Duncombe, W. G. 1964. The colorimetric micro-determination of non-esterified fatty acids in plasma. *Clin. Chim. Acta.* **9**: 122–125.
33. Fletcher, M. J. 1968. A colorimetric method for estimating serum triglycerides. *Clin. Chim. Acta.* **22**: 393–397.
34. Olsen, C. 1971. An enzymatic fluorimetric micromethod for the determination of acetoacetate, β-hydroxybutyrate, pyruvate and lactate. *Clin. Chim. Acta.* **33**: 293–300.
35. Bremer, J., and E. J. Davis. 1972. Phosphorylation coupled to acyl-coenzyme A dehydrogenase-linked oxidation of fatty acids by liver and heart mitochondria. *Biochim. Biophys. Acta.* **275**: 298–301.
36. Herrara, E., and N. Freinkel. 1968. Interrelationships between liver composition, plasma glucose and ketones, and hepatic acetyl-CoA and citric acid during prolonged starvation in the male rat. *Biochim. Biophys. Acta.* **170**: 244–253.
37. Aas, M., and L. N. W. Daae. 1971. Fatty acid activation and acyl transfer in organs from rats in different nutritional states. *Biochim. Biophys. Acta.* **239**: 208–216.
38. Bieber, L. L., T. Abraham, and T. Helmrath. 1972. A rapid spectrophotometric assay for carnitine palmitoyltransferase. *Anal. Biochem.* **50**: 509–518.
39. Meier, J. M., J. D. McGarry, G. R. Faloona, R. H. Unger, and D. W. Foster. 1972. Studies of the development of diabetic ketosis in the rat. *J. Lipid Res.* **13**: 228–233.