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Fatty Acid Oxidation, Citric Acid Cycle Activity, and Morphology of Mitochondria in Diabetic Rat Liver* (34008)

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Fatty acid oxidation and citric acid cycle activity in the liver are thought to be important factors in ketogenesis. Either increased breakdown of fatty acid (FA) or impairment of citric acid cycle activity may cause ketonemia, but the mechanism regulating these two systems has not been adequately clarified. Insulin has been found to spare the oxidation of intravenously injected palmitate-1-^{14}C when measured in diabetic rat liver slices. (1). In other studies, no significant increase was demonstrated in the oxidation of palmitate-1-^{14}C when added directly to slices and isolated mitochondria (MT) from diabetic rat liver (2, 3). These conflicting reports warrant further studies in isolated MT where FA oxidation primarily takes place.

In this paper we report that increases in palmitate and oleate oxidation in isolated MT are accompanied by changes in the ultrastructure of MT. Metabolic and structural changes in MT of diabetic rat liver were reversed by insulin treatment. The activities of citric acid cycle enzymes were not altered in MT of diabetic rat liver.

Materials and Methods. Diabetes was induced in male Wistar rats weighing 150-250 g by intravenous injection of alloxan monohydrate, 6 mg/100 g body wt. Rats with blood sugar exceeding 250 mg/100 ml were used. MT were isolated by a modification of Schneider's method (4). After initial centrifugation of the homogenate at 620*g* for 10 min to remove the nuclear fraction, the supernatant was centrifuged at 13,000*g* for 10 min. After washing once in 0.25 *M* sucrose containing 0.5 m*M* EDTA at pH 7.5, MT were suspended in twice as much sucrose solution as the original fresh liver weight. Protein determinations in MT and supernatant fractions were done by the biuret method (5).

FA oxidation was determined according to a modification of Bjorntorp's method (6). The rate of incorporation of radioactivity in $^{14}CO_2$ and ^{14}C acetoacetate from ^{14}C FA was found to be proportional to the amount of MT suspension added under the following test conditions. The assay system (3 ml) contained (in micromoles) sucrose, 100; $MgCl_2$, 10; phosphate buffer (pH 7.5), 40; ATP, 6; glucose, 50; FA, 0.3 (containing 0.04–0.06 μCi of palmitate-1(U)-^{14}C or oleate-U-^{14}C and bovine albumin in ratio of FA/albumin=7), hexokinase, 6 units; cytochrome *c*, 0.39 mg, and 0.4 ml of MT suspension.

Materials for ultrastructural study (pellets of isolated MT or biopsy specimens from the left lateral lobe of the liver) were fixed in ice cold veronal-buffered 2% osmium tetroxide (7) for 1 hr, progressively dehydrated through graded alcohols, and embedded in

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TABLE I. Long-Chain FA Oxidation in Isolated MT: Percentage of Recovery of $^{14}C \pm SD/0.2$ g Liver/30 min at 37°.[a]

Substrate	Product	Normal	Diabetic	Diabetic + insulin
Pal-1-^{14}C	CO_2	1.05 ± 0.16 (5)	2.08 ± 0.69 (5)[b]	0.69 ± 0.33 (5)
	Acetoacetate	0.46 ± 0.1 (3)	1.77 ± 0.84 (5)[b]	0.24 ± 0.08 (4)
Oleate-U-^{14}C	CO_2	0.60 ± 0.17 (3)	2.20 ± 0.66 (3)[b]	0.31 ± 0.14 (3)
	Acetoacetate	0.20 ± 0.05 (3)	0.66 ± 0.17 (3)[b]	0.19 ± 0.09 (3)

[a] Number of rats in parentheses. Diabetic: 1–2 weeks; insulin: 10–15 units of Lente/100 g body wt for 3 days.

[b] By *t* test, diabetic *vs.* normal, $p < .025$; when recovery in acetoacetate was calculated, carboxyl-^{14}C was multiplied by 2 in case of pal-1-^{14}C and by 4 in case of oleate-U-^{14}C.

Maraglas (8). Ultrathin sections were cut on an LKB ultratome, stained with uranyl acetate and lead citrate, and viewed in an RCA EMU 3 G electron microscope. The size of MT was measured by a point-counting technique (9).

The activities of citric acid cycle enzymes were measured spectrophotometrically (10) in the 13,000*g* supernatant fraction and in isolated MT suspension after freeze-thawing three times and sonication. Alpha-ketoglutarate dehydrogenase (α-KGDH) was measured in intact MT by adding Lubrol WX (11). Molar extinction coefficients (A_{240}) for aconitate and fumarate were taken to be 3.3×10^3 $cm^{-1}M^{-1}$ and 2.4×10^3 cm^{-1} M^{-1} (12). Succinate dehydrogenase (SDH) was measured by the reduction rate of potassium ferricyanide (A_{400}). Linear dependencies of activities could be demonstrated according to the increasing amount of the sample in each reaction and the values expressed as V_{max}.

Results. FA oxidation. The rate of oxidation of palmitate-1-^{14}C to CO_2 and acetoacetate was doubled over normal and that of oleate-U-^{14}C tripled in MT from diabetic rat liver. These increases were not observed after insulin treatment (Table I). In the presence of DL-carnitine (1.33 m*M*), the sum of ^{14}C incorporation into CO_2 and acetoacetate from palmitate-U-^{14}C was also increased by 50% in MT of diabetic rat liver (normal: 12.2 ± 3.8% at 37°/0.2 g/30 min; 2–4 week diabetes: 18.7 ± 2.24, $p < .01$). Elevation of palmitate oxidation first occurred on Day 2 after alloxan administration and became maximal by Day 7 to 8; a similar pattern was noted for oleate, but the maximum level was reached on Day 6.

Morphology of isolated MT and MT within hepatocytes. Isolated MT from diabetic rat liver when examined by electron microscopy appeared to be denser and larger than normal. Measurement of the longer diameter of isolated MT from diabetic rat liver demonstrated a 20–40% increment (normal: 0.75 ± 0.15 μ, diabetic: 1.02 ± 0.18μ, $p < .001$). Ultrastructural study of MT in biopsy specimens revealed that the MT in the hepatocytes of diabetic rats were remarkably enlarged (Fig. 1). Branched and atypical MT were frequently observed. The average size of MT was found to be increased by 60% while the number of MT per cut section of a cell was not changed in diabetes (Table II). Insulin treatment reversed these morphologic alterations. Enlargement of the MT was first noted on Day 2 after alloxan injection; maximal enlargement occurred on Day 6. The enlargement of MT approximately coincided with the elevation of LCFA oxidation by isolated MT.

Activities of citric acid cycle enzymes. NAD-specific isocitrate dehydrogenase (ICDH), α-KGDH, and citrate synthase displayed the lowest activites of the enzymes tested both in MT and supernatant fraction (Table III). With the exception of supernatant fumarase, which exhibited 100% increase over normal, no changes in activities were observed in the diabetic liver. The overall activity of the cycle was studied by succinate-1, 4-^{14}C oxidation and no changes were observed in MT from diabetic rat liver.

Discussion. Since oleate and palmitate

TABLE II. Calculated Size (μ^2) and Relative Area of MT in Hepatocytes.[a]

Rats	No. of rats	Total MT counted	Average MT size $\mu^2 \pm$ SD (1)	Ratio of total MT area /cell area $\pm$ SD (2)
Normal	8	1725	0.56 ± 0.07	0.14 ± 0.02
Diabetic	9	2438	0.91 ± 0.31[b]	0.22 ± 0.05[c]
Diabetic + insulin	2	500	0.67 ± 0.06	0.16 ± 0.014

[a] At least two micrographs were measured from each rat at magnification 8220×. Regularly plotted points, 0.5 cm apart, were superimposed on the micrograph and total points (A) falling inside of MT, total points (B) per cell area, and number (C) of MT were determined. (1) was calculated from A/C, (2) from A/B; insulin was given for 3 days after 7 days of diabetes; diabetic: 4–32 days.

[b] $p < .01$.

[c] $p < .001$, diabetic *vs.* normal.

comprise approximately 60% of serum nonesterified FA (13), from which ketone bodies are mainly derived, the increased activity of long-chain (LC) FA oxidation in diabetic liver MT may play a significant role in the formation of ketone bodies. The absence of significant impairment of the activity of the citric acid cycle in MT from diabetic rats supports this concept.

The morphologic correlate of increased LCFA oxidation appears to be enlargement of hepatic MT. The temporal coincidence of MT enlargement and increased LCFA in MT of diabetic rat liver, along with reversal of both the biochemical and morphologic changes by insulin, are strong evidence for this suggestion. Hepatic MT enlargement has been reported in rats treated with cortisone acetate for 6 days (14). In contrast to the findings in the present study, in which the relative area of MT within hepatic cells was increased, cortisone treatment in rats caused a decrease in the number of MT within the cell with no alteration in total MT area and volume.

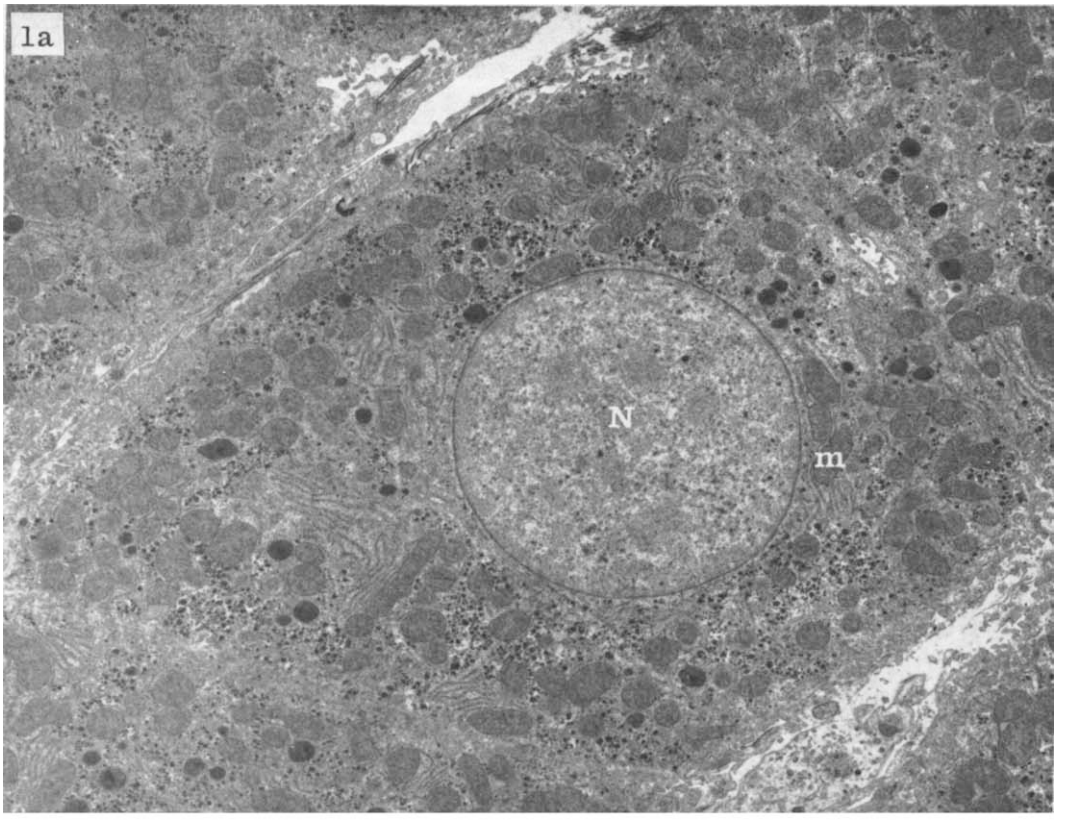
In studies currently in progress, enlargement of MT and elevation of LCFA oxidation have been observed in adrenalectomized alloxan-diabetic rats. It is, therefore, probable that the changes observed in hepatic MT are due to insulin deficiency rather than an adrenal hormonal effect. Further study is

TABLE III. Activities of Citric Acid Cycle Enzymes (V_{max}) in Normal and Diabetic Rat Liver (μmoles substrates/g fresh liver/min $\pm$ SD at 25°).[a]

Enzyme	No. of rats	Supernatant	Mitochondria	Total
Citrate synthase	5	0.23 ± 0.12	0.86 ± 0.21	1.09 ± 0.24
Aconitase	9	6.11 ± 0.91	1.05 ± 0.51	7.17 ± 1.11
ICDH (NADP)	6	16.48 ± 3.16	3.54 ± 1.23	20.02 ± 4.25
(NAD)	4	0	0.17 ± 0.09[c]	
α-KGDH	4	0	0.27 ± 0.15	0.27 ± 0.15
SDH	7	1.06 ± 0.28	2.71 ± 0.85	3.77 ± 1.08
Fumarase				
Normal	7	16.31 ± 4.10	5.55 ± 0.86	21.86 ± 3.44
Diabetic	5	35.76 ± 9.38[b]	5.20 ± 1.39	40.96 ± 8.09[b]
MDH	11	284.32 ± 44.04	89.85 ± 29.2	374.17 ± 66.67

[a] With the exception marked by [b], enzyme activities in the diabetic rat liver (4–32 days) were not significantly different at the 5% level from normal values listed above. One g of liver yields 130–140 mg of protein in the supernatant and 25–30 mg in MT fraction from normal and diabetic rat liver. MDH: malate dehydrogenase.

[c] Measured without adding ADP.


1a
N
m

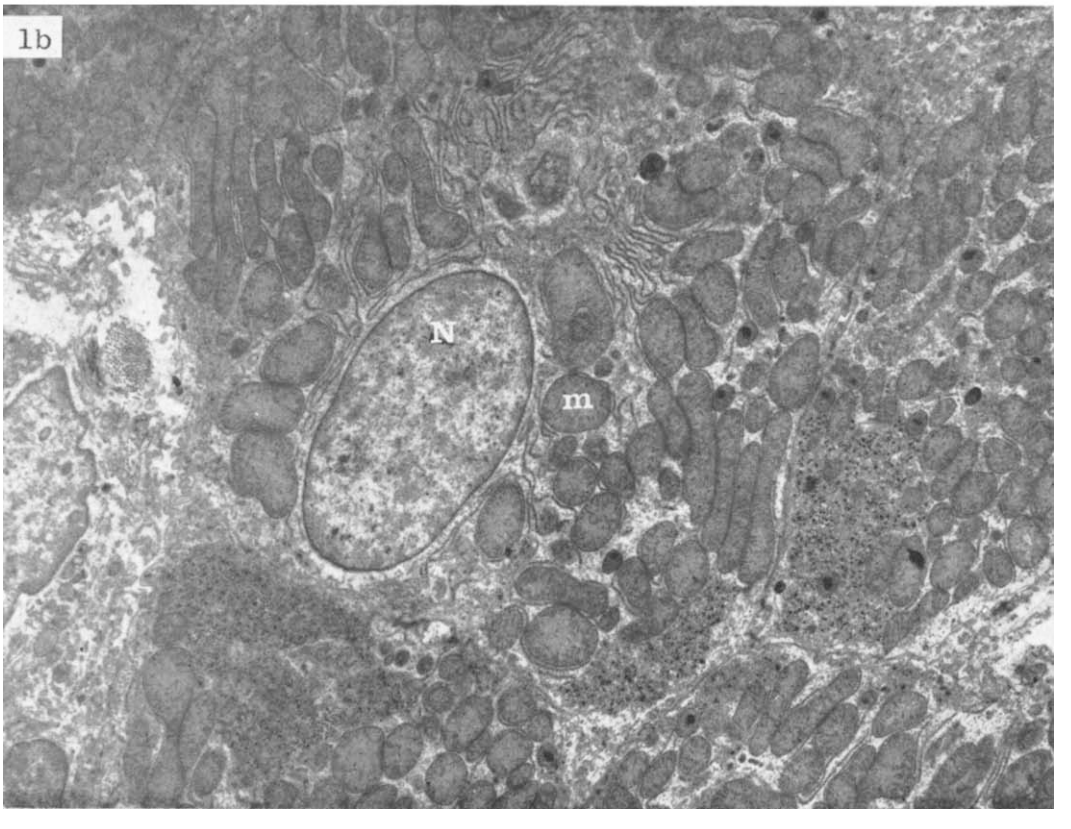

1b
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FIG. 1. a. Normal rat liver, 4700X. N = nucleus. M. = mitochondrion. b. Liver, alloxan diabetic rat, 10 days, 4700X. Note enlarged, atypical mitochondria.

necessary to elaborate the relationship between MT enlargement and LCFA oxidation and to define the steps responsible for the observed increase in LCFA oxidation in diabetes. Carnitine-palmityl transferase has been suggested to be rate-limiting for LCFA oxidation (15). It may be significant that the activity of this enzyme has been reported to be twice as active in the MT fraction of diabetic rat liver (16).

Summary. Palmitate and oleate oxidation in isolated mitochondria from alloxan diabetic rat liver were shown to be elevated two or threefold over normal. These increases were accompanied by enlargement of mitochondria as observed by electron microscopy. These metabolic and structural changes were reversed by treating the diabetic rat with insulin. The activities of citric acid cycle enzymes in MT were unchanged in the diabetic state. It is suggested that insulin regulates LCFA oxidation in the liver by an effect on MT structure and function.

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