

[¹⁴C]Palmitate uptake in isolated rat liver mitochondria: effects of fasting, diabetes mellitus, and inhibitors of carnitine acyltransferase

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Abstract The rapid association of Na-[16-¹⁴C]palmitate with isolated rat liver mitochondria was measured by an oil separation method. This association was time and temperature-dependent and was absolutely dependent on the presence of exogenous ATP and CoASH and partially dependent on exogenous carnitine. Carnitine dependence was enhanced at lower concentrations of [¹⁴C]palmitate. At 6.5 μM [¹⁴C]palmitate (molar ratio of palmitate to albumin equal to 0.54), the rate of association was linear for 20 sec and was increased more than 100% in the presence of carnitine. Carnitine-dependent association was inhibited by 2-bromopalmitate, an inhibitor of carnitine acyltransferase I, but not by (+)-octanoylcarnitine, a presumed inhibitor of carnitine acyltransferase II. The association of [¹⁴C]palmitate with mitochondria was enhanced from 190 to 330% in mitochondria isolated from fasted animals and from 160 to 230% in mitochondria isolated from diabetic, ketotic animals as compared to control animals. The enhanced association with mitochondria from fasted animals was inhibited by 2-bromopalmitate. These studies demonstrate a method of evaluating fatty acid association with mitochondria which, because of its dependence on carnitine and carnitine acyltransferase I activity, most likely represents true uptake into mitochondria. Furthermore, these studies indicate that the carnitine-dependent uptake of fatty acids into mitochondria is enhanced in the two ketotic states evaluated and that the carnitine acyltransferase system may be a regulatory site in ketone body production.

Supplementary key words fatty acid uptake · ketone bodies

Enhanced ketogenesis can be dissociated from elevated circulating levels of FFA. In diabetic man, physiologic elevations of plasma glucagon augment ketone body production above that observed at similar FFA levels in the absence of glucagon (1). Also, at any level of FFA delivered to the liver, perfused livers from diabetic or fasted animals produce more ketone bodies than livers from nondiabetic, fed animals (2, 3). Livers from normal animals perfused with glucagon also exhibit enhanced ketone body production (4–6). More recently, McGarry, Wright, and Foster (7) have

shown that administration of either glucagon or anti-insulin serum to rats *in vivo* alters the “ketogenic set” of the liver; a “nonketogenic” liver is changed to a “ketogenic” liver during subsequent perfusion with fatty acids *in vitro*. In the case of glucagon, this change can occur prior to an increase in circulating FFA. These observations suggest that intracellular regulatory factors could lead to increased ketone body production. Such factors include extramitochondrial processes such as decreased triglyceride and/or fatty acid synthesis (8–10). They also include intramitochondrial changes such as activation of carnitine acyltransferase (3, 11–13), an enzyme system necessary for the uptake of long chain FFA into mitochondria, alterations in the citric acid cycle (9, 13, 14), and changes in the ratio of acetyl CoA/CoASH (15, 16).

The role of the mitochondria in regulating ketogenesis has not been precisely defined. Recently much interest has been directed toward the carnitine acyltransferase system and consequent FFA uptake into mitochondria as a possible regulatory site of ketone body production. Despite mounting evidence that this system regulates ketone body production in the isolated, perfused liver (3, 7), it is uncertain whether carnitine acyltransferase activity is increased in ketogenic states. While some investigators have reported enhanced activities in the ketosis of diabetes (11, 17) and fasting (11, 12, 18), others have been unable to reproduce these findings (3) or have attributed the apparent increases in activity to the method of cal-

Abbreviations: FFA, free fatty acids; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; CoASH, coenzyme A; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid; CAT I, carnitine acyltransferase I; CAT II, carnitine acyltransferase II.

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ulation (19). Furthermore, the reported increase in the maximal rate of palmitate oxidation to acetoacetate in mitochondria isolated from livers of ketotic animals (20) is also controversial (19). It is clear, however, that fasting (3), diabetes (2), and acute changes in glucagon or insulin (7) can alter the ketogenic capacity of the liver, most likely at the acylcarnitine transferase reaction.

In the present studies, we describe a method for the direct measurement of carnitine-responsive FFA uptake in isolated rat liver mitochondria and present the first direct evidence that fatty acid uptake is enhanced in mitochondria isolated from ketotic animals.

MATERIALS AND METHODS

Male Sprague-Dawley rats, fed ad libitum and weighing approximately 200 g, were used for all experiments. Diabetes was induced by a single injection of alloxan (60 mg/kg) via the left femoral vein under light ether anesthesia. Twenty-four hours later the animals were begun on 2–4 units of PZI insulin daily; after 10–14 days the insulin was discontinued. Those animals that exhibited glycosuria of at least 20 mg/ml and “moderate” ketonuria (Keto-Diastix, Ames Company, Elkhart, IN), usually within 96 hr after insulin withdrawal, were killed by a sharp blow to the head. Control animals were matched for age and weight at the time the experimental animals received alloxan. At the time of killing, the control animals weighed 294 ± 8 g (mean \pm SEM) and the diabetic rats weighed 243 ± 8 g. The 48-hr-starved animals and their controls weighed 200 g initially and 178 ± 8 g and 217 ± 1.6 g, respectively, at the time of killing.

The method of isolation of rat liver mitochondria has been described previously (14) and is essentially that of Schnaitman and Greenawalt (21). The isolated mitochondria were suspended in a 2 mM HEPES–220 mM mannitol–70 mM sucrose solution, pH 7.4, containing 0.5 mg of fatty acid-poor bovine serum albumin per ml to a concentration ranging from 5.8 to 17 mg mitochondrial protein/ml.

Uptake of [$^{16-14}\text{C}$]palmitate was assessed by a modification of the method of Werkheiser and Bartley (22) as follows. Mitochondria (0.12–0.49 mg of protein) were added at 24°C to an incubation medium, pH 7.4, containing 2 mM HEPES, 10 mM KH_2PO_4 , 100 mM KCL, 3 mM MgCl_2 , 40 mM sucrose, 0.33 mM ADP, and 0.1 mM L-malate in a total volume of 0.33 ml. The incubation was supplemented with 3 mM ATP, 40 μM reduced CoASH, and either 1 mM L- or DL-carnitine as indicated. L-Carnitine and the racemic mixture were equally effective in enhancing fatty acid uptake at 1

mM concentrations. The reactions were initiated by the addition of the indicated amounts of Na-[$^{16-14}\text{C}$]palmitate complexed to albumin (molar ratio of 0.54 at 6.5 μM and 0.96 at 16.3 μM [^{14}C]palmitate) and the mixture was agitated. It was found that the molar ratio of [^{14}C]palmitate to albumin is critical, for at higher ratios the rapidity of uptake makes it difficult to measure increases in uptake with time. Palmitate uptake was terminated by transferring the incubation mixture to a Beckman microfuge tube containing 0.1 ml of a 4:1 mixture (d 1.027 g/ml) of dibutylphthalate (d 1.043 g/ml)/dinonylphthalate (d 0.98 g/ml) oils and centrifuging at 14,000 g for 45 sec in a Beckman microfuge. During centrifugation the mitochondria sedimented through the oil and were separated from the incubation medium. Since a mitochondrial pellet is visible within 4 sec of centrifugation, the incubation was considered terminated at the instant centrifugation began. Following centrifugation, the microfuge tube was cut below the interphase between the medium and oil. The portion containing the mitochondrial pellet was placed in 0.3 ml of 1 M KOH. After heating to dissolve the pellet, 10 ml of scintillation fluid (Instabray) was added and the radioactivity was determined using a Beckman liquid scintillation counter. Unless indicated, uptake as reported represents the total [^{14}C]palmitate associated with the mitochondria in the presence of ATP, CoASH, and carnitine minus the [^{14}C]palmitate associated with mitochondria in the absence of ATP, CoASH, and carnitine. This procedure automatically corrects for the amount of [^{14}C]palmitate trapped outside the mitochondria in the mitochondrial pellet. Uptake measured at 20 and 45 sec is linear up to 3.0 mg mitochondrial protein/ml and for [^{14}C]palmitate concentrations of 1.6–8 μM .

The quantity of mitochondrial protein sedimented by our method was the same in the presence or absence of the oil mixture for centrifugations of 45 sec and 4 min. The adequacy of centrifugation was further evaluated by a modification of the method of Rodbell (23). Identical microfuge tubes with and without oil were centrifuged at 9,700 g for 15 min in a Sorvall refrigerated centrifuge used for the preparation of mitochondria. The microfuge tubes were placed in rubber adaptors which were filled to the top of the microfuge tube with water to prevent deformation of the tube. Under these conditions, the mitochondrial protein sedimented is the same as that sedimented following a 14,000 g centrifugation for 45 sec in the Beckman microfuge.

The sodium salts of [$^{16-14}\text{C}$]palmitate and 2-bromopalmitate were prepared according to Goodman (24). In experiments using Na-2-bromopalmitate and (+)-

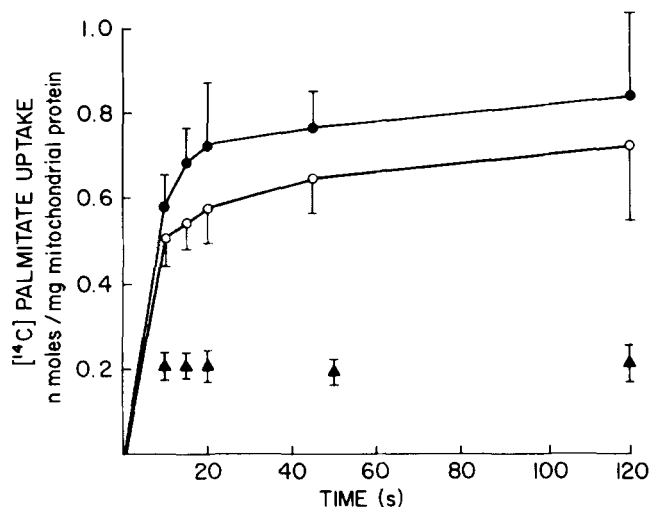


Fig. 1. Uptake of Na-[16-¹⁴C]palmitate (16.3 μ M) in isolated rat liver mitochondria in the presence of ATP, CoASH, and carnitine (●), in the presence of ATP and CoASH (○), and in the absence of these additions (▲). Uptake was measured at 24°C as described in the Methods section in the presence of 16.3 μ M [¹⁴C]palmitate complexed to albumin in a molar ratio of 0.96. Uptake in the presence of carnitine is significantly different from uptake in the absence of carnitine by paired *t* analysis ($P < 0.05$ at 2 min, $P < 0.01$ at 45 sec, and $P < 0.005$ at all other times). The values represent the mean \pm SEM of duplicate determinations from four separate experiments at 120 sec and 10 separate experiments at all other times.

octanoylcarnitine, the mitochondria were preincubated in the presence and absence of the transport inhibitors for 5 min at 24°C in incubation medium supplemented with ATP, CoASH, and carnitine prior to the addition of Na-[16-¹⁴C]palmitate.

The inulin space was measured by the addition of carboxyl-[¹⁴C]inulin to mitochondria as described for the measurement of [¹⁴C]palmitate uptake. It is recognized that the inulin space most likely overestimates the trapping of extramitochondrial water (22); in these studies, however, it is unnecessary to know the exact amount trapped since the method of calculation of uptake automatically corrects for this. Mitochondrial protein was measured either by the method of Lowry et al. (25) when HEPES was absent or by the Biuret method (26).

L-Carnitine hydrochloride and (+)-octanoylcarnitine were a gift from the Otsuka Pharmaceutical Co., Osaka, Japan. [16-¹⁴C]Palmitic acid (53–59 mCi/mmol) and carboxy-[¹⁴C] inulin were purchased from New England Nuclear, Boston, MA; ATP (disodium salt) and L-malic acid from Sigma, St. Louis, MO; reduced coenzyme A (free acid), and HEPES from Calbiochem, San Diego, CA; DL-carnitine·HCl from Aldrich, Milwaukee, WI; fatty acid-poor bovine serum albumin (lot number 50) from Pentex Biochemicals, Kankakee, IL; Instabray from Yorktown, New Hyde

Park, NY; and alloxan monohydrate from J. T. Baker, Phillipsburg, NJ.

RESULTS

The uptake of [16-¹⁴C]palmitate at 24°C by isolated rat liver mitochondria is illustrated in **Fig. 1**. In the absence of ATP, CoASH, and carnitine, and in the presence of 16.3 μ M Na-[¹⁴C]palmitate, the [¹⁴C]palmitate associated with the mitochondria did not change from 10 sec to 2 min of incubation and represented 0.2 nmol [¹⁴C]palmitate/mg mitochondrial protein. This fraction most likely represents the sum of trapping plus nonspecific adhesion of the fatty acid to mitochondrial membranes. Under the same conditions as Fig. 1 and at 0.53 mg mitochondrial protein/ml, the inulin space was equal to 0.14% of the total FFA added. With the addition of ATP and CoASH, uptake was enhanced 150–250% and increased, al-

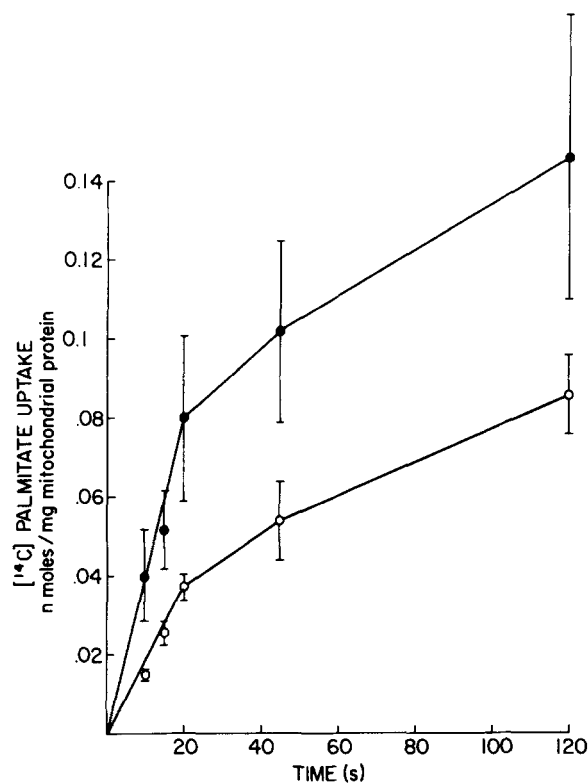


Fig. 2. Uptake of Na-[16-¹⁴C]palmitate (6.5 μ M) in the presence of ATP, CoASH and carnitine (1 mM) (●) and in the presence of ATP and CoASH alone (○). Uptake was measured as described in the legend to Fig. 1 after the addition of 6.5 μ M [¹⁴C]palmitate complexed to albumin (molar ratio, 0.54). The uptake in the absence of ATP, CoASH, and carnitine was 0.11 ± 0.02 nmol/mg mitochondrial protein and has been subtracted from all points. The data represent the mean \pm SEM of duplicate determinations of 10–15 separate experiments. By paired *t* test $P < 0.05$ at 10 sec, $P < 0.02$ at 15 sec, and $P < 0.01$ at 20 sec, 45 sec, and 120 sec.

though not linearly, with time. The addition of 1.0 mM DL-carnitine enhanced uptake further. In other experiments under these conditions, the palmitate uptake was enhanced maximally at 1.0 mM DL-carnitine with enhancement of 2.6, 38, and 56% of maximal at 0.05, 0.1, and 0.5 mM DL-carnitine, respectively.

Since the oxidation of palmitate in rat liver mitochondria is increasingly dependent upon the presence of carnitine as the concentration of palmitate is decreased (27), we measured uptake in the presence of a lower concentration of [14 C]palmitate, 6.5 μ M. Under these conditions, uptake was linear to 20 sec and the magnitude of enhancement by carnitine was substantially larger, from 170% at 10 sec to 70% at 2 min (Fig. 2).

In experiments not shown, the temperature dependence of uptake was evaluated following a 45-sec incubation at 6.5 μ M [14 C]palmitate. At 4°C the uptake of [14 C]palmitate ranged from 17 to 35% of the uptake at 24°C. At 37°C, uptake was 180–280% of that at 24°C. Intact mitochondria were necessary for maximal uptake. Mitochondria disrupted by repeated freeze-thawing had uptake from 0 to 0.006 nmol/mg mitochondrial protein per 45 sec (less than 10% of intact mitochondria) and displayed no enhancement with the addition of carnitine.

2-Bromopalmitate is reported to be a specific inhibitor of fatty acid oxidation (28) and CAT I (28–30). To further characterize the uptake of [14 C]palmitate by the oil separation method, we preincubated mito-

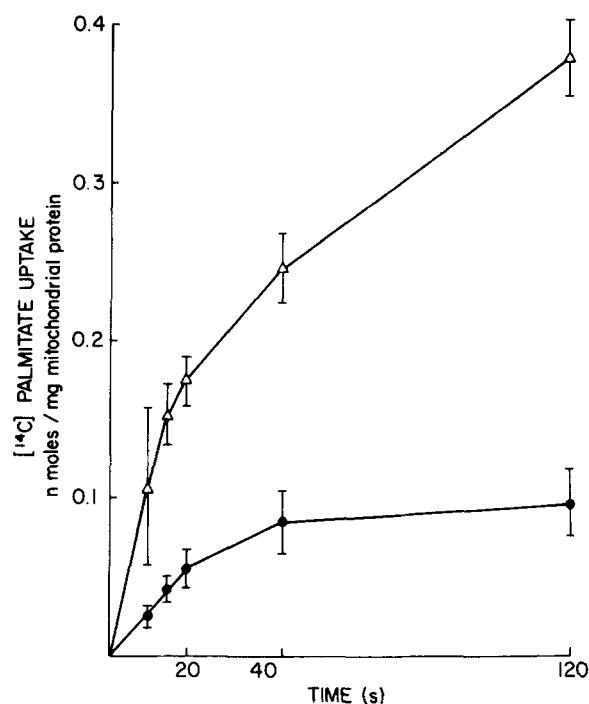


Fig. 3. The uptake of [14 C]palmitate (6.5 μ M) in the presence of ATP, CoASH, and carnitine by liver mitochondria isolated from control (●) and 48-hr-fasted (Δ) rats. Uptake was measured as described in the Methods section in the presence of 0.26–0.43 mg of mitochondrial protein and 0.16–0.49 mg of mitochondrial protein from control and fasted animals, respectively. The values represent the mean \pm SEM of duplicate determinations from four separate experiments. With the exception of the 10-sec points, all values are significantly different by paired *t* test ($P < 0.01$).

TABLE 1. Effect of 2-bromopalmitate on [14 C]palmitate uptake in rat liver mitochondria

Additions	nmol uptake/45 sec per mg mitochondrial protein			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Control ^a	0.043	0.032	0.064	0.041
DL-Carnitine ^b (1 mM)	0.100	0.115	0.092	0.088
DL-Carnitine (1 mM) + Na-2-Bromo- palmitate (μ M) ^c				
1.0	0.049	0.079	0.047	0.027
0.5	0.069	0.093	0.063	
0.25	0.063	0.104		

^a Control mitochondria were suspended in the incubation medium described in the Methods section supplemented with 3 mM ATP and 40 μ M CoASH.

^b By paired *t* test, uptake in the presence of carnitine is significantly greater than in the absence of carnitine, $P < 0.02$.

^c Mitochondria were preincubated with 2-bromopalmitate as described in the Methods section prior to the addition of 6.5 μ M [14 C]palmitate. By paired *t* test the inhibition of uptake is significant at 1 μ M, $P < 0.01$, and 0.5 μ M, $P < 0.01$, 2-bromopalmitate.

chondria in the presence of 2-bromopalmitate prior to assessing [14 C]palmitate uptake. As indicated in **Table 1**, 2-bromopalmitate, 1 μ M, inhibited carnitine-dependent uptake at 6.5 μ M [14 C]palmitate. In experiments 1 and 2, 1 μ M 2-bromopalmitate inhibited carnitine-dependent uptake 89% and 43%, respectively. However, in experiments 3 and 4, 1 μ M 2-bromopalmitate decreased uptake to below that observed in the absence of carnitine. This leads us to believe that a portion of the uptake observed in the absence of exogenously added carnitine may be due to endogenous carnitine which has not been removed by washing the mitochondria. Carnitine itself enhanced uptake by more than 100% in three of four experiments (Table 1). At lower concentrations of 2-bromopalmitate, there was progressively less inhibition of uptake (Table 1).

We next evaluated the contribution of CAT II to the observed carnitine-dependent uptake through the use of (+)-octanoylcarnitine, an agent thought to inhibit CAT II (31, 32). Unlike bromopalmitate, (+)-octanoylcarnitine (1 mM) did not inhibit [14 C]palmitate uptake in the presence of 0.5 or 1.0 mM DL-carnitine. This was true when mitochondria were preincubated with

TABLE 2. Effect of 2-bromopalmitate on [¹⁴C]palmitate uptake in mitochondria from fasted animals

Additions	nmol uptake/45 sec per mg mitochondrial protein		
	Exp. 1	Exp. 2	Exp. 3
Control ^a	0.076	0.041	0.162
DL-Carnitine ^b (1 mM)	0.25	0.176	0.240
DL-Carnitine (1 mM) + Na-2-Bromo- palmitate (μ M) ^c			
1.0	0.073	0.052	0.157
0.5	0.142	0.075	0.198

^a See footnote a to Table 1.

^b By paired *t* test, uptake in the presence of carnitine is significantly greater than in the absence of carnitine, $P < 0.05$.

^c See footnote c to Table 1. By paired *t* test, the inhibition of uptake is significant at 1 μ M, $P < 0.05$; but not 0.5 μ M 2-bromopalmitate.

(+)-octanoylcarnitine for 5 min at 24°C and 37°C (data not shown). Since these data suggest that differences in uptake in the presence as compared to the absence of carnitine are an indication of CAT I and not CAT II activity, this technique might be useful in evaluating metabolic states with altered CAT I activity. Previous studies in perfused rat liver indicate that fasting and diabetes mellitus may be two such states (2, 3).

A comparison of FFA uptake in mitochondria from 48-hr-fasted and control rats is shown in Fig. 3. In four experiments in the presence of carnitine, the mean [¹⁴C]palmitate uptake in mitochondria isolated from 48-hr-fasted animals was 290–430% of that in mitochondria isolated from control animals. The enhanced uptake in mitochondria from fasted animals is carnitine-dependent (Table 2) and, as for uptake in mitochondria from control animals (Table 1), is inhibited by Na-2-bromopalmitate (Table 2). In two of three experiments in the absence of added carnitine, uptake was similar in mitochondria isolated from control (Table 1) and fasted (Table 2) animals.

Enhanced carnitine acyltransferase activity has also been suggested in the ketosis associated with diabetes mellitus (7, 33). As shown in Fig. 4, [¹⁴C]palmitate uptake in mitochondria from ketotic diabetic rats was 260–330% of the [¹⁴C]palmitate uptake in mitochondria from control animals.

DISCUSSION

These studies demonstrate the feasibility of measuring fatty acid uptake in isolated rat liver mitochondria.

The uptake is absolutely dependent upon the presence of ATP and CoASH (Fig. 1) and partially dependent upon the addition of carnitine (Fig. 2, Table 1). The work of Chase and Tubbs (28), as well as our own (data not shown), indicates that 2-bromopalmitate inhibits the carnitine-dependent oxidation of palmitate and palmitoyl-CoA, but not the oxidation of palmitoyl-carnitine, by liver mitochondria. These findings as well as additional studies by Chase and Tubbs using the CoASH and carnitine esters of 2-bromopalmitate (28) and the studies of West, Chase, and Tubbs (29) and Yates and Garland (30) have been interpreted as indicating that 2-bromopalmitoyl-CoA formed from 2-bromopalmitate inactivates a pool of carnitine palmitoyltransferase accessible to external acyl-CoA (CAT I) but not a second pool of carnitine palmitoyltransferase inaccessible to added acyl-CoA (CAT II). In the present studies, 2-bromopalmitate decreases [¹⁴C]palmitate uptake in the presence of carnitine (Tables 1, 2). The uptake measured is not inhibited by (+)-octanoylcarnitine, an agent thought to

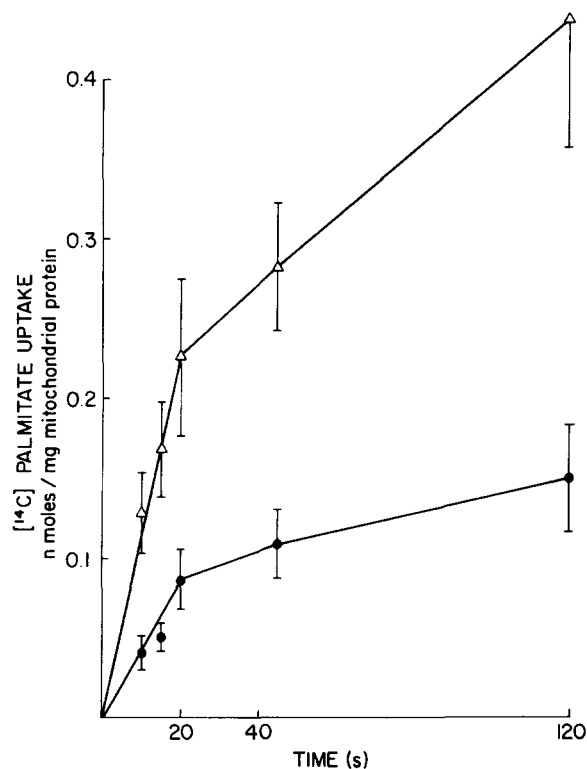


Fig. 4. Uptake of Na-[¹⁴C]palmitate by liver mitochondria from control (●) and alloxan diabetic rats (Δ). Uptake was measured under the conditions described in the legend to Fig. 3 in the presence of 0.28 mg of mitochondrial protein and 0.19–0.30 mg of mitochondrial protein from control and diabetic animals, respectively. The values represent the mean \pm SEM of duplicate determinations from eight separate mitochondrial preparations. By paired *t* test $P < 0.005$ at 15 sec and 45 sec, $P < 0.01$ at 10 sec and 20 sec, and $P < 0.05$ at 120 sec.

inhibit CAT II (31). Under similar conditions (+)-octanoylcarnitine inhibits [¹⁴C]palmitate oxidation by 62%.² As suggested by Hoppel (34), however, it remains to be established that the effects of acyl-D-carnitine derivatives on fatty acid oxidation and ketone body production are due primarily to effects on acylcarnitine transferase.

The usefulness of this oil separation method to measure fatty acid uptake in mitochondria from rats with altered metabolic states is illustrated by the finding that mitochondria from both fasted and diabetic ketotic animals exhibit enhanced rates of palmitate uptake (Table 2, Figs. 3 and 4). This enhanced uptake is totally dependent on carnitine in two of three experiments and partially dependent on carnitine in a third (Table 2). The inhibition by bromopalmitate (Tables 1, 2) shows the importance of CAT I in the stimulation of uptake by carnitine (Table 1) and in the enhanced uptake in fasted animals (Table 2). From these studies it seems likely that CAT I activity is enhanced in fasted and diabetic animals. However, it is difficult to draw conclusions as to the role of CAT II.

Enhanced fatty acid oxidation and ketone body production in perfused livers from fasted and diabetic rats (2, 3) have been attributed to enhanced activity of CAT II (3). Enhanced CAT II activity has been reported in liver homogenates and isolated mitochondria from fasted and diabetic animals (11, 12, 17). In microsomal fractions from fasted animals (18) and mitochondrial fractions from diabetic animals (17), enhanced activity of CAT I has been observed. Others, however, have not confirmed increased CAT I or II activity and have indicated that previously observed changes might not be real (19).

Harano et al. (17) have reported decreased respiratory control, an increased maximum rate of palmitate oxidation to acetoacetate, and ultrastructural alterations in mitochondria isolated from diabetic, ketotic rats (20). All of these alterations were reversed by insulin therapy. Interestingly, while Harano et al. (20) observed a two-fold increase in palmitate oxidation in mitochondria from ketotic rats, there was no increase in palmitoylcarnitine oxidation. This suggests that CAT I may be the responsible step for enhanced fatty acid oxidation and agrees with our data revealing an enhanced fatty acid uptake that is dependent on CAT I activity.

We have not directly measured CAT activity; however, we have demonstrated a method of evaluating fatty acid association with mitochondria that is dependent upon CAT I activity (Tables 1, 2). This as-

sociation is enhanced during diabetes mellitus and fasting when expressed per milligram of mitochondrial protein and lends further support to the concept that the CAT system may be a major site for the control of ketogenesis. As discussed previously, increases in fatty acid uptake would lead to a disproportionate enhancement of ketone body production (14).

It is not possible at this time to state whether the increase in fatty acid uptake in mitochondria from fasted and diabetic animals is a primary alteration due to changes in the hormonal milieu or a secondary alteration due to the altered intracellular milieu. In our laboratory we have been unable to demonstrate direct effects of cyclic nucleotides on fatty acid metabolism in isolated rat liver mitochondria (14). Fatty acids, however, in addition to altering the acetyl CoA/CoASH ratio and Krebs cycle activity as suggested by others, may enhance the carnitine-dependent transport of [¹⁴C]palmitate into mitochondria (14). This suggests that the enhanced uptake observed in mitochondria from diabetic and fasted animals may have been induced at least in part by increased intracellular FFA levels. Alternatively, as suggested by others, the glycogen content of the liver may play a role in the regulation of carnitine acyltransferase activity (7). At present, however, the mechanism by which the ketogenic profile and the enhanced capacity for fatty acid uptake occur is unknown. ■■

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