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Hepatic mitochondrial inner membrane properties and carnitine palmitoyltransferase A and B

Effect of diabetes and starvation

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Intact mitochondria and inverted submitochondrial vesicles were prepared from the liver of fed, starved (48 h) and streptozotocin-diabetic rats in order to characterize carnitine palmitoyltransferase kinetics and malonyl-CoA sensitivity *in situ*. In intact mitochondria, both starved and diabetic rats exhibited increased $V_{max.}$, increased K_m for palmitoyl-CoA, and decreased sensitivity to malonyl-CoA inhibition. Inverted submitochondrial vesicles also showed increased $V_{max.}$ with starvation and diabetes, with no change in K_m for either palmitoyl-CoA or carnitine. Inverted vesicles were uniformly less sensitive to malonyl-CoA regardless of treatment, and diabetes resulted in a further decrease in sensitivity. In part, differences in the response of carnitine palmitoyltransferase to starvation and diabetes may reside in differences in the membrane environment, as observed with Arrhenius plots, and the relation of enzyme activity and membrane fluidity. In all cases, whether rats were fed, starved or diabetic, and whether intact or inverted vesicles were examined, increasing membrane fluidity was associated with increasing activity. Malonyl-CoA was found to produce a decrease in intact mitochondrial membrane fluidity in the fed state, particularly at pH 7.0 or less. No effect was observed in intact mitochondria from starved or diabetic rats, or in inverted vesicles from any of the treatment groups. Through its effect on membrane fluidity, malonyl-CoA could regulate carnitine palmitoyltransferase activity on both surfaces of the inner membrane through an interaction with only the outer surface.

INTRODUCTION

Carnitine palmitoyltransferase (CPT) activity exists on both sides of the mitochondrial inner membrane, and each activity has catalytically different properties (Hoppel & Tomec, 1972; Bieber & Farrell, 1983; Hoppel & Brady, 1985). CPT-A has been proposed to function as the rate-limiting enzyme in hepatic mitochondrial β-oxidation of long-chain fatty acids (McGarry & Foster, 1980). Its activity changes with alterations in concentrations of acyl-CoA species and malonyl-CoA (McGarry & Foster, 1980), and with changes in nutritional (Bremer, 1981; Saggerson *et al.*, 1982, 1984; Cook, 1984) and hormonal (Harano *et al.*, 1972; Saggerson & Carpenter, 1981; Stakkestad & Bremer, 1983; Cook *et al.*, 1984) state. Little attention has focused on the CPT activity of the inner surface of the inner membrane *in situ*, the 'CPT-B'.

Fiol & Bieber (1984) have found that the two CPT activities may arise from a single protein, at least in ox heart. It appears, then, that specific characteristics of the two activities may result from specific relationships to their membrane environments (Bergstrom & Reitz, 1980; Clarke & Bieber, 1981; Bieber & Farrell, 1983; Brady & Brady, 1985). Further, malonyl-CoA has been proposed to regulate the balance of fatty acid synthesis and oxidation, at least in liver, through its inhibition of CPT-A (McGarry & Foster, 1980). Sensitivity to malonyl-CoA is decreased in starvation (Bremer, 1981; Cook, 1984) and in diabetes (Cook *et al.*, 1984). Other data suggest that the malonyl-CoA interaction with CPT-A activity is mediated by a receptor distinguishable from the CPT active site (Mills *et al.*, 1984). In the fed state, the effect of malonyl-CoA may be exerted, in part, by alterations in the fluidity of the mitochondrial inner membrane (Brady & Brady, 1985), which could co-ordinate control of CPT-A and CPT-B activities.

In the present studies, we have investigated the effects of starvation and diabetes on kinetic parameters, malonyl-CoA sensitivity and response of CPT activity to temperature perturbation in intact mitochondria and inverted submitochondrial vesicles.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats were obtained from the Washington State University colony or from Zivic–Miller (Allison Park, PA, U.S.A.), and maintained on chow and water *ad libitum*. The rats were housed in group cages. In the starvation studies, food was withdrawn 48 h before they were killed. To induce diabetes, animals were injected via the tail vein with 60–110 mg of strepto-

Abbreviations used: CPT, carnitine palmitoyltransferase; CPT-A and CPT-B, the activity on the outer and on the inner surface of the inner mitochondrial membrane respectively [conventions of Hoppel (1982) and Kiorpes *et al.* (1984)]; DPH, diphenylhexatriene.

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Table 1. Kinetic parameters and malonyl-CoA sensitivity for hepatic carnitine palmitoyltransferase activity of intact mitochondria and inverted submitochondrial vesicles

Intact mitochondria and inverted submitochondrial vesicles were prepared as described in the Experimental section. CPT activity was determined at 30 °C by using a 2 min incubation. The reaction mixture contained 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 1 mg of defatted dialysed bovine serum albumin (fraction V)/ml, 4 mg of sodium tetrathionate/ml, 10–60 μM-palmitoyl-CoA, 0.01–2.0 mM-L-carnitine containing L-[*Me*-^{14}C]carnitine, pH 7.0, in a final volume of 0.25 ml. The reaction was initiated by the addition of 50 μg of mitochondria or submitochondrial-vesicle protein. K_i was calculated from Dixon plots by using 10 μM- and 40 μM-palmitoyl-CoA and 0.5 mM-L-carnitine. Malonyl-CoA concentration was varied from 0 to 50 μM (0, 5, 10, 25, 50 μM). Data were calculated by the method of Segel (1975). Each value is the mean ± S.E.M. for three observations. Statistically significant effects are designated with an asterisk (*).

Mitochondria	Fed	Starved	Diabetic
Intact			
$V_{max.}$ (nmol/min per mg)	10.6±4.4*	20.5±5.9	25.6±5.4
K_m (carnitine) (mM)	0.6±0.2	0.4±0.2	0.9±0.2
K_m (palmitoyl-CoA) (μM)	19±3*	42±19	35±11
K_i (malonyl-CoA) (μM)	10.5±3.8*	23.2±8.0	24.2±8.6
Inverted vesicles			
$V_{max.}$ (nmol/min per mg)	24.3±4.7	33.1±6.7	48.8±6.7
K_m (carnitine) (mM)	0.3±0.1	0.2±0.1	0.4±0.1
K_m (palmitoyl-CoA) (μM)	54±11	42±14	34±14
K_i (malonyl-CoA) (μM)	76.3±7.0	70.2±8.5	114.0±8.5

zotocin/kg body wt in 0.05 M-sodium citrate (pH 4.0) after starvation overnight. The rats were maintained on insulin for 10 days after the streptozotocin injection, after which period insulin was withdrawn, and ketosis was allowed to develop (Brady *et al.*, 1982). Urine glucose and ketones were monitored with commercial test sticks. Only rats showing significant glucosuria and ketonuria within 48 h of insulin withdrawal were used.

Mitochondrial isolation and incubation

This procedure has been described in detail (Hoppel *et al.*, 1979). In brief, rats were killed by decapitation, and livers placed into ice-cold buffer (220 mM-mannitol, 70 mM-sucrose, 5 mM-Mops, 2 mM-EDTA, pH 7.4). Livers were rinsed, blotted, weighed, minced and homogenized with a loose-fitting Teflon pestle. Mitochondria were then isolated by differential centrifugation. The mitochondrial pellet was washed twice, and diluted to a final concentration of approx. 50 mg of protein/ml (Gornall *et al.*, 1949).

Inverted submitochondrial vesicles

These were prepared from the fresh mitochondria by using nitrogen compression/decompression as described by Fleischer *et al.* (1974). The inverted vesicles sedimented as brownish translucent pellets. Success of the inversions was determined by measuring the ratio of succinate oxidase to succinate:cytochrome *c* reductase, and by determination of D-β-hydroxybutyrate dehydrogenase (Vidal *et al.*, 1983). In a series of four liver mitochondria inversions, the succinate oxidase/succinate:cytochrome *c* reductase ratio was 18±3 when measured by the methods described, with the modification that activities were measured in iso-osmotic buffer at 30 °C. Such a ratio indicates approx. 90% efficiency of the inversion. The β-hydroxybutyrate dehydrogenase activity was undetectable in intact mitochondria in iso-osmotic buffer; in inverted vesicles at 30 °C, the activity was similar to that observed by Vidal *et al.* (1983). This serves to confirm further a high efficiency of inversion. The success of inversion, as measured by these ratios, did not differ with treatment, and Vidal *et al.* (1983) have previously compared inverted submitochondrial vesicles prepared from liver mitochondria of fed and diabetic rats. For subsequent kinetic analyses, it was necessary to use inverted vesicles on the day of preparation to avoid loss of activity.

Assay methods

CPT activity was measured in the direction of palmitoylcarnitine formation with L-[*Me*-^{14}C]carnitine as substrate (Hoppel & Tomec, 1972). The CPT assay, pH 7.0, contained, in a final volume of 0.25 ml: 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 1 mg of dialysed defatted bovine serum albumin/ml, 4 mg of sodium tetrathionate/ml, 5–60 μM-palmitoyl-CoA, 0.01–2.0 mM total L-carnitine (unlabelled plus ^{14}C-labelled). After a 5 min preincubation, the reaction was initiated by the addition of 50 μg of mitochondrial protein, and the reaction allowed to proceed for 2 min at 30 °C for the kinetic determinations unless otherwise noted. The specific radioactivity of the L-[*Me*-^{14}C]carnitine was adjusted as necessary to provide adequate sensitivity. Sodium tetrathionate was added to prevent the back reaction (to serve as a CoASH sink). The assay was linear for up to 4 min with 100 μg of protein. Kinetic parameters were estimated by varying both palmitoyl-CoA (10 and 40 μM) and carnitine (0.01–2 mM at each of the palmitoyl-CoA concentrations). Initial plots of reciprocal velocity versus reciprocal carnitine concentration were replotted to give slope and intercept as a function of reciprocal palmitoyl-CoA concentration. The kinetic parameters were derived from the secondary plots as detailed by Segel (1975). Dixon plots were generated by using 10 μM- and 40 μM-palmitoyl-CoA, 0.5 mM-L-carnitine and 0, 5 μM-, 10 μM-, 25 μM- and 50 μM-malonyl-CoA (Segel, 1975). Activity measured in the presence of malonyl-CoA was linear during the period measured and exhibited correlation coefficients of 0.99 or better. The resulting Dixon plots were also linear.

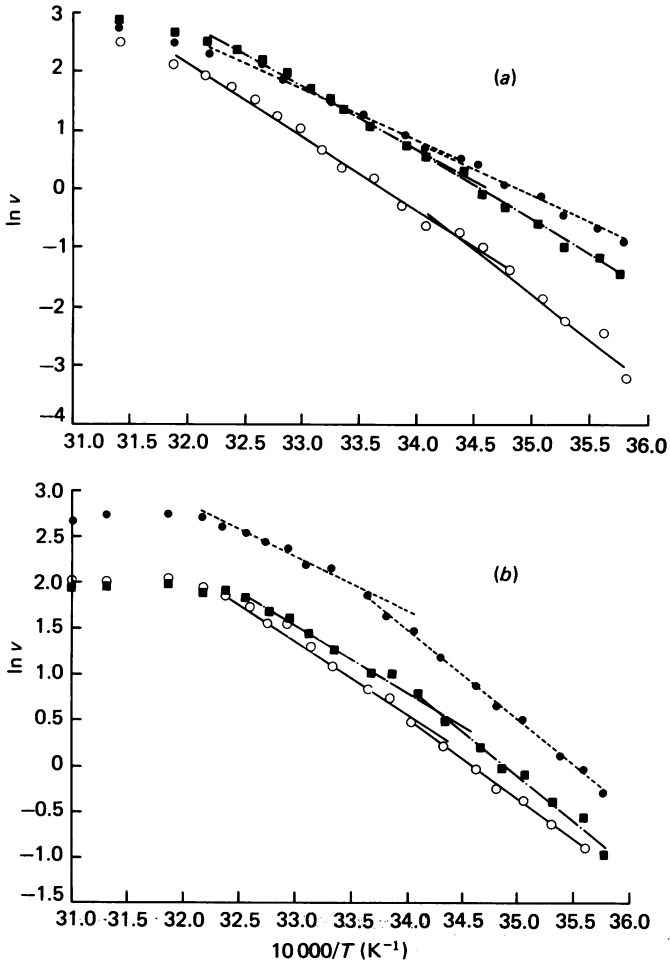

Fig. 1. Effects of temperature on activity of CPT in intact mitochondria (*a*) and in inverted submitochondrial vesicles (*b*)

Hepatic mitochondria (*a*) and submitochondrial vesicles (*b*) were prepared from fed (○), 48 h-starved (●) and diabetic (■) rats. Energies of activation (E_a) were calculated for two curve segments by using linear-regression analysis of ln [*v* (nmol/min per mg)] as a function of $10000/T$. E_a for the upper segment refers to the range approx 31–34 on the abscissa, with the lower segment greater than 34. In the intact mitochondria (*a*), E_a values were: fed, 21.9 and 31.6; starved, 15.2 and 18.7; diabetic, 19.9 and 24.1. Standard errors were 1.2 and 2.2 ($n = 3$). In the inverted submitochondrial vesicles, E_a values were: fed, 12.1 and 19.3; starved, 11.7 and 20.4; diabetic, 11.7 and 19.3; with standard errors of 0.9 and 1.1 ($n = 3$).

Arrhenius plots were generated over a temperature range of 6–50 °C, with 40 μM-palmitoyl-CoA and 0.5 mM-carnitine in the standard assay configuration. Energy of activation, E_a, was calculated from the slope of the plot of ln (activity) versus $1/T$, where slope $= E_a/R$ (Williams & Williams, 1972). We have previously found that the K_m for neither palmitoyl-CoA nor carnitine is temperature-dependent (Brady & Brady, 1985).

Fluorescence polarization was determined with DPH as probe (Prendergast *et al.*, 1981; Stubbs *et al.*, 1984). The concentration of mitochondrial or vesicle protein was adjusted to optimize the signal-to-noise ratio (5–10 mg of protein/ml). Polarization was determined spectrophotofluorimetrically (Perkin–Elmer model MFP-3L) with 366 nm excitation and 426 nm emission wavelengths, by using polarizing filters in both excitation and emission planes.

Significance of treatment effects were determined by analysis of variance with a completely randomized design (Steel & Torrie, 1960). Statistical significance was set at the 5% level.

Materials

Streptozotocin was given by the Upjohn Co., Kalamazoo, MI, U.S.A. L-Carnitine was given by Sigma Tau, Rome, Italy. The L-[*Me*-^{14}C]carnitine was synthesized by the method of Ingalls *et al.* (1982), with the modification that demethylcarnitine was concentrated by freeze-drying, which provides adequate dryness to give crystals. Palmitoyl-CoA was synthesized as described by Seubert (1960). Sodium tetrathionate was obtained from K & K Laboratories, Plainview, NY, U.S.A. Bovine serum albumin was prepared by defatting (Chen, 1967) and dialysing (Hanson & Ballard, 1968) commercially available albumin (fraction V). DPH was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Malonyl-CoA was obtained from P-L Biochemicals (Piscataway, NJ, U.S.A). All other reagents were reagent grade or better.

RESULTS AND DISCUSSION

CPT activity is found on both sides of the mitochondrial inner membrane. At least in heart, these activities may be the product of a single protein (Fiol & Bieber, 1984). To the extent that differences exist between CPT activity on the outer surface and inner surface, these differences must arise as a function of the membrane environment. It becomes critical to study CPT activity *in situ* if the role of membrane location is to be understood. Our work with the inverted submitochondrial vesicle indicated that valid comparison can be made between the 'inner' activity (represented by the vesicles) and intact mitochondria, which express the 'outer' activity (Brady & Brady, 1985). Several investigators have examined the effect of starvation and/or diabetes on the kinetics and malonyl-CoA sensitivity of CPT (Bremer, 1981; Cook *et al.*, 1984; Cook, 1984; Saggerson *et al.*, 1984). Table 1 presents the data for CPT kinetics in intact mitochondria and inverted submitochondrial vesicles derived in the present studies. In the intact mitochondria, $V_{max.}$ was increased and the malonyl-CoA sensitivity decreased with starvation and diabetes, which is consistent with the findings of the previously mentioned studies. The K_m for carnitine was somewhat higher in the diabetic rats, but unchanged in the starved animals, compared with the fed state. The apparent K_m for palmitoyl-CoA increased 2–3-fold with both starvation and diabetes. These data differ from those of Saggerson & Carpenter (1981) and Cook (1984), where no change was observed with starvation. The reason for this difference is unclear; however, it should be noted that the assay of CPT and the mathematical analysis applied are not strictly comparable with those presented here.

The inverted submitochondrial vesicles showed increases in $V_{max.}$ with both diabetes and starvation. Further, the estimated $V_{max.}$ was approximately twice that for the intact mitochondria. To our knowledge, no study has clearly defined the degree of association of either CPT activity to its membrane. During the process

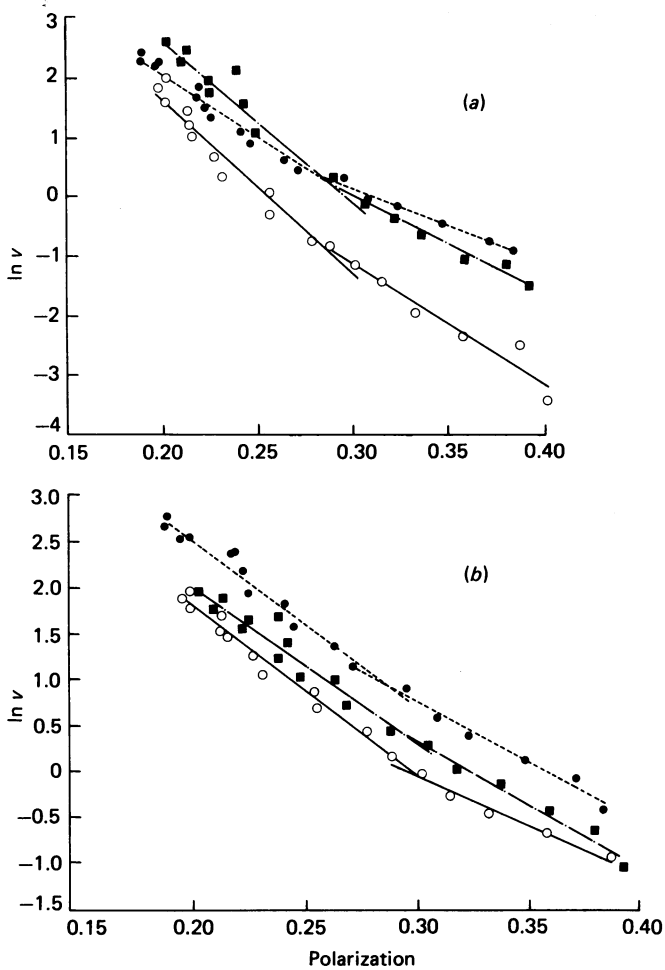

Fig. 2. CPT velocity as a function of membrane polarization

CPT activity, expressed as ln [v (nmol/min per mg)], was assessed as a function of membrane polarization, by using a DPH probe, in intact mitochondria (*a*) and inverted submitochondrial vesicles (*b*) in fed (○), 48 h-starved (●) and diabetic (■) rats. Lines were calculated by linear-regression analysis.

Table 2. Response of hepatic mitochondrial membrane DPH polarization to pH and malonyl-CoA in fed and starved rats

Mitochondria were isolated as described in the Experimental section. The mitochondria were either used immediately or frozen at −80 °C before use. Mitochondria (0.5–1.0 mg) were suspended in 2 ml of 80 mM-KCl/50 mM-Mops/1 mM-EGTA adjusted to the desired pH with KOH. Membranes were preincubated for 30 min with DPH in tetrahydrofuran (100 nmol/2 ml), followed by addition of malonyl-CoA (final concn. 100 μM). Fluorescent polarization was determined by using an excitation wavelength of 366 nm and an emission wavelength of 426 nm. Each value is the mean of three observations. Standard errors were approx. 0.014.

			Polarization				
Rats	Treatment	pH...	6.6	6.8	7.0	7.2	7.4
Fed	None						
	Fresh		0.285	0.258	0.275	0.253	0.238
	Frozen		0.190	0.182	0.231	0.227	0.212
	100 μM-Malonyl-CoA						
	Fresh		0.307	0.328	0.314	0.272	0.256
	Frozen		0.204	0.166	0.179	0.198	0.201
Starved	None						
	Fresh		0.224	0.193	0.182	0.186	0.173
	Frozen		0.187	0.195	0.190	0.178	0.187
	100 μM-Malonyl-CoA						
	Fresh		0.216	0.193	0.171	0.184	0.192
	Frozen		0.172	0.192	0.191	0.188	0.187
Diabetic	None						
	Fresh		0.177	0.200	0.188	0.154	0.220
	Frozen		0.175	0.168	0.174	0.177	0.176
	100 μM-Malonyl-CoA						
	Fresh		0.183	0.184	0.181	0.167	0.191
	Frozen		0.176	0.183	0.181	0.182	0.186

of preparing the inverted vesicles, a quantity of CPT activity is released to the supernatant (soluble plus bound to small membrane fragments) equal to that recovered in inverted vesicles. It is not possible to differentiate CPT-A and -B activity in the supernatant, for obvious reasons. It may be that the 'inner' activity, CPT-B, is not strongly attached to the inner membrane. It could exist in equilibrium between free and bound activity. If this were so, we would fail to detect it with the present approach.

In contract with the CPT-A activity, the CPT-B activity showed no change in K_m for palmitoyl-CoA or carnitine as a function of treatment. The K_i for malonyl-CoA was considerably (3–6-fold) higher for inverted vesicles for all treatment groups when compared with intact mitochondria. We have previously found that the inverted vesicles are less sensitive to malonyl-CoA, but do retain a degree of sensitivity (Brady & Brady, 1985). The present study confirms this finding.

Two approaches were used to examine the relationship of CPT activity to membrane environment. The first approach was to examine the effects of temperature perturbation of the membrane on CPT activity (Fig. 1). The Arrhenius plots for both intact mitochondria and inverted vesicles could be resolved into curves of two segments, with heat-inactivation occurring above 40 °C. The calculated energies of activation for both starved and diabetic animals differed from those for the fed animals, depending on the mitochondrial preparation. In the intact hepatic mitochondria, where CPT $V_{max.}$ and K_m for palmitoyl-CoA were increased by both starvation and diabetes, E_a values calculated from the lower segment were decreased by both treatments. In contrast, in the inverted vesicles, where no real changes were observed in K_m for either palmitoyl-CoA or carnitine with treatment, no changes in the E_a calculated from either the upper or the lower segment were observed. This is consistent with different lipid environments for the two activities. To examine this further, ln (CPT activity) was plotted as a function of DPH fluorescence polarization. DPH serves as a probe for non-polar lipid, and should reflect the deep membrane environment (Prendergast *et al.*, 1981; Stubbs *et al.*, 1984). The relationship is non-linear (Fig. 2), and seems to be equally well described as a quadratic function or as two linear functions much as the Arrhenius plot (as presented here). The region corresponding to polarization of 0.2–0.3 is the temperature range 40–25 °C. Decreasing polarization is interpreted as increasing fluidity. Thus, for both intact mitochondria and inverted vesicles, CPT

activity increased as the membrane became more fluid. The relationship between CPT activity and fluidity differed both with treatment and with preparation. In particular, the intact mitochondria from starved rats had a slope for both curve segments less than that for either fed or diabetic rats. In the inverted vesicles, all three treatments yielded parallel lines.

In all cases, increasing membrane fluidity was associated with increasing activity in both inverted and intact preparations. Further, both starvation and diabetes appeared to result in decreased polarization (Table 2), which would indicate increased membrane fluidity. This phenomenon may contribute to the increased activity observed in these states. Vidal *et al.* (1983) have correlated changes in fatty acid composition of phospholipid in diabetes with breaks in the Arrhenius plot for β-hydroxybutyrate dehydrogenase, although they could not correlate these with bulk fluidity changes as measured with DPH.

Malonyl-CoA has been previously found to decrease fluidity of the mitochondrial inner membrane when presented to the outer surface, but not when presented to the inner surface (Brady & Brady, 1985). Since increased activity of both CPT-A and CPT-B is associated with increased fluidity of the membrane (Fig. 2), this interaction of malonyl-CoA with the membrane could serve to regulate the activity of both CPT-A and CPT-B concomitantly. In the present study, the relationship of malonyl-CoA to membrane fluidity as a function of pH was examined with starvation in both fresh and frozen intact mitochondria (Table 2) and inverted vesicles (results not shown). The inverted vesicles showed no consistent response to malonyl-CoA in either fresh or frozen preparations with any of the physiological states examined.

The pH of the medium has been found to influence CPT-A sensitivity to malonyl-CoA inhibition, with greatest sensitivity below pH 7.2 (Mills *et al.*, 1984). With fresh mitochondria from fed rats, malonyl-CoA causes the membrane to become more rigid (increasing polarization). As shown in Fig. 2, increasing rigidity is associated with decreased CPT activity. This effect was lost if the mitochondria are frozen before use. However, in the starved or diabetic rats, the effect does not appear to exist. In either fresh or frozen mitochondria from starved or diabetic rats, malonyl-CoA did not cause increased rigidity. This observation is consistent with the decrease in sensitivity to malonyl-CoA inhibition observed in both of these states. However, the starved- and diabetic-rat mitochondria retain some sensitivity, although decreased, to malonyl-CoA. The loss of the malonyl-CoA fluidity effect with these states may reflect the limitations of a generalized fluidity probe such as DPH. Alternatively, the fluidity effect may represent only one aspect of the interaction of malonyl-CoA with CPT. Mitochondria from fed and starved rats exhibited similar increases in fluidity with ethanol and benzyl alcohol (results not shown), indicating that the mitochondria from the starved rats were capable of a fluidity response. Again, the lack of response to malonyl-CoA could reflect the limitations of the DPH probe.

In using a probe, such as DPH, only gross changes in a particular membrane environment are monitored. Changes in microenvironments are overlooked. The microenvironmental changes may ultimately be the more significant. It is tempting to attribute the effects of malonyl-CoA on membrane fluidity to interaction with its putative receptors, particularly because such a mechanism would provide co-ordinated control of CPT activity on both sides of the mitochondrial inner membrane. Further study will be required before the importance of malonyl-CoA effects on fluidity of the mitochondrial membrane relative to fatty acid oxidation can be defined.

Note added in proof (Received 20 September 1985)

If inverted vesicles are further purified by washing with cytochrome *c*–Sepharose, the K_i values for malonyl-CoA are increased 2–3-fold, presumably owing to removal of contaminating right-side-out vesicles (L. J. Brady & P. S. Brady, unpublished work).

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REFERENCES

Bergstrom, J. D. & Reitz, R. (1980) Arch. Biochem. Biophys. **204**, 71–79
Bieber, L. L. & Farrell, S. O. (1983) Enzymes 3rd Ed. **16**, 627–644
Brady, P. S. & Brady, L. J. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 1415 (abstr. 5873)
Brady, P. S., Schumann, W. C., Ohgaku, S., Scofield, R. F. & Landau, B. R. (1982) J. Lipid Res. **23**, 1317–1320
Bremer, J. (1981) Biochim. Biophys. Acta **665**, 628–631
Chen, R. F. (1967) J. Biol. Chem. **242**, 173–181
Clarke, P. R. H. & Bieber, L. L. (1981) J. Biol. Chem. **256**, 9861–9868
Cook, G. (1984) J. Biol. Chem. **259**, 12030–12033
Cook, G., Stevens, T. W. & Harris, R. (1984) Biochem. J. **219**, 337–339
Fiol, C. J. & Bieber, L. L. (1984) J. Biol. Chem. **259**, 4164–4170
Fleischer, S., Meissner, G., Smigel, M. & Wood, R. (1974) Methods Enzymol. **31**, 292–299
Gornall, A. G., Bardawill, C. J. & David, M. (1949) J. Biol. Chem. **177**, 751–766
Hanson, R. W. & Ballard, F. J. (1968) J. Lipid Res. **9**, 667–668
Harano, Y., Kowal, J., Yamazaki, R., Lavine, L. & Miller, M. (1972) Arch. Biochem. Biophys. **153**, 426–437
Hoppel, C. L. (1982) Fed. Proc. Fed. Am. Soc. Exp. Biol. **41**, 2853–2857
Hoppel, C. L. & Brady, L. J. (1985) in The Enzymes of Biological Membranes (Martonosi, A., ed.), pp. 139–175, Plenum Press, New York
Hoppel, C. L. & Tomec, R. J. (1972) J. Biol. Chem. **247**, 832–844
Hoppel, C. L., DiMarco, J. P. & Tandler, B. (1979) J. Biol. Chem. **254**, 4164–4170
Ingalls, S., Hoppel, C. L. & Turkaly, J. S. (1982) J. Labelled Compd. Radiopharm. **9**, 535–541
Kiorpes, T. C., Hoerr, D., Ho, W., Weaner, L. E., Inman, M. G. & Tutwiler, G. F. (1984) J. Biol. Chem. **259**, 9750–9755
McGarry, J. D. & Foster, D. W. (1980) Annu. Rev. Biochem. **49**, 395–420
Mills, S. E., Foster, D. W. & McGarry, J. D. (1984) Biochem. J. **219**, 601–608
Prendergast, F., Haugland, R. & Callahan, P. (1981) Biochemistry **20**, 7333–7338

Saggerson, E. D. & Carpenter, C. A. (1981) FEBS Lett. **129**, 225–228
Saggerson, E. D., Carpenter, C. A. & Tselentis, B. S. (1982) Biochem. J. **208**, 667–672
Saggerson, E. D., Bird, M. I., Carpenter, C. A., Winter, K. A. & Wright, J. J. (1984) Biochem. J. **224**, 201–206
Segel, I. H. (1975) Enzyme Kinetics, John Wiley and Sons, New York
Seubert, W. (1960) Biochem. Prep. **9**, 80–83
Stakkestad, J. A. & Bremer, J. (1983) Biochim. Biophys. Acta **280**, 244–252
Steel, R. G. D. & Torrie, J. H. (1960) Principles and Procedures of Statistics, pp. 99–131, McGraw-Hill Co., New York
Stubbs, C., Kinosita, K., Munkonge, F., Quinn, P. & Ikegami, A. (1984) Biochim. Biophys. Acta **775**, 374–380
Vidal, J. C., McIntyre, J. O., Churchill, P., Andrew, J. A., Peheut, M. & Fleischer, S. (1983) Arch. Biochem. Biophys. **224**, 643–658
Williams, H. & Williams, V. (1972) Physical Biochemistry for the Life Sciences, pp. 313–321, W. H. Freeman, San Francisco

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