Hepatic carnitine palmitoyltransferase turnover and translation rates in fed, starved, streptozotocin-diabetic and diethylhexyl phthalate-treated rats

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Hepatic carnitine palmitoyltransferase (CPT) turnover was studied in control and in non-ketotic hyperglycaemic streptozotocin-diabetic rats. The degradation constant (k_d) and half-life ($t_{1/2}$) did not appear to be altered by mild diabetes. The hepatic CPT ($\mu\text{g/g}$ of liver) was not increased by the mild, non-ketotic, diabetes. However, the total hepatic CPT ($\mu\text{g/liver}$) was 37% greater in the diabetic animals, owing to the increased liver weight. This resulted from a 40% increase in the synthesis constant (k_s). Hepatic CPT activity (total detergent-solubilized) and translation rates were measured in fed, starved (48 h), non-ketotic diabetic, ketotic diabetic and diethylhexyl phthalate (DEHP)-treated rats. CPT activity (munits/mg of mitochondrial protein) was not significantly increased with non-ketotic diabetes (44% increase, but non-significant), but was increased approx. 2-fold with starvation and ketotic diabetes, and 3.5-fold with DEHP treatment. CPT expressed as units/liver was increased non-significantly (23%) in non-ketotic and starved rats, similar to the turnover study, but was significantly increased with ketotic diabetes and with DEHP treatment. mRNA-translation activity for CPT was elevated in all states to a somewhat greater extent than was activity. It was concluded that protein synthesis as a product of increased CPT-mRNA translation activity is a major means of long-term regulation.

INTRODUCTION

Carnitine palmitoyltransferase-A (CPT-A) has been proposed as the rate-limiting enzyme in the hepatic mitochondrial β -oxidation of long-chain fatty acids (McGarry & Foster, 1980). The activity of hepatic CPT-A is generally increased in diabetes in experimental animals, although the method of induction of diabetes may influence the result (Brady *et al.*, 1985; Harano *et al.*, 1972a; Nosadini *et al.*, 1979; Saggerson & Carpenter, 1981). Likewise, there is generally a decrease in the sensitivity of CPT-A to malonyl-CoA inhibition in experimental diabetes (Brady *et al.*, 1985; Cook *et al.*, 1984; Gamble & Cook, 1985). We have shown that the sensitivity to inhibitors and kinetic parameters of purified CPT are altered by the lipid environment or water activity *in vitro* (Brady *et al.*, 1985; Brady & Brady, 1986, 1987). The magnitude of change in purified or partially purified CPT activity which can be produced *in vitro* by manipulating the lipid environment, or by using dimethyl sulphoxide or octyl glucoside (Brady & Brady, 1986, 1987; Pande *et al.*, 1986; Woldegiorgis *et al.*, 1985; Bremer *et al.*, 1985) approximates the degree of observed increase in CPT-A activity *in situ* (2–4-fold) with diabetes (Brady *et al.*, 1985; Brady & Brady, 1987; Harano *et al.*, 1972a; Nosadini *et al.*, 1979).

Harano *et al.* (1982) have shown that the CPT-A activity of mitochondria isolated from hepatocytes increases in response to glucagon and decreases in response to insulin, but the mechanism was not studied. The same group later suggested that glucagon addition

to isolated hepatocytes leads to CPT phosphorylation (Harano *et al.*, 1985). Thus the question arises as to whether the amount of CPT protein actually increases in diabetes, or whether short-term regulation is predominant, whether via modification of existing protein, via alteration in sensitivity to malonyl-CoA, or via changes in the lipid or protein micro-environment of CPT in the inner membrane. To address this question, the present study was designed to determine the turnover of hepatic CPT in fed and non-ketotic diabetic rats and the specific mRNA-translation rates in fed, starved, non-ketotic diabetic, ketotic diabetic and diethylhexyl phthalate-treated rats.

EXPERIMENTAL

Animals

CPT was purified from liver of adult (> 300 g) male Sprague-Dawley rats obtained from the Washington State University breeding colony. CPT was induced by feeding them on a commercial stock diet with 2% diethylhexyl phthalate (DEHP) for 2–4 weeks before they were killed. Polyclonal antibody was raised in female BALB/c mice also obtained from Washington State University.

For the remainder of the studies, male weanling Sprague-Dawley rats were used (Washington State University breeding colony). Animals were fed on a commercial stock diet (Purina Chow; Ralston Purina, St. Louis, MO, U.S.A.) for 2–3 weeks before the initiation

Abbreviations used: CPT, carnitine palmitoyltransferase (activities are located on both sides of the mitochondrial inner membrane: CPT-A refers to that on the outer surface, and CPT-B to that on the inner surface); DEHP, diethylhexyl phthalate.

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of studies. After 2 weeks acclimatization to our animal facility, DEHP-treated rats were fed on the stock diet with 2% (w/w) DEHP for 2 weeks before being killed. Non-ketotic and ketotic diabetic rats were prepared after 3 weeks acclimatization by injection of streptozotocin via the tail vein. Non-ketotic rats received 55 mg of streptozotocin/kg body wt., whereas ketotic rats received 110 mg/kg body wt., 1 week before being killed (Brady *et al.*, 1982). Routine checks of urine during the study with Keto-Diastix (Ames Laboratories, Elkhart, IN, U.S.A.) revealed that glucosuria, but not ketonuria, was occurring in most of the non-ketotic rats. Those not showing glucosuria were removed from the study. It must be stressed that the injection of 55 mg of streptozotocin/kg body wt. produces a mild diabetic state. In the absence of insulin treatment, most of these animals may be expected to recover euglycaemia within a few weeks. This is further demonstrated by the non-significant weight difference between these animals and the controls over the week of the study. The ketotic diabetic rats exhibited a more severe diabetic state. It was necessary to inject these animals with insulin periodically during the week before killing them. The dose of insulin was adjusted to control the ketonuria, with no effort made to attain euglycaemia. Even with insulin therapy, approx. 1 in 5 of these animals will die, presumably owing to the acute streptozotocin toxicity. Insulin was withdrawn, and those rats developing ketonuria within 48 h were used. Starved rats were starved for 48 h before being killed (plasma [acetoacetate] = 0.87 ± 0.20 mM; mean $\pm$ S.E.M., $n = 4$). Average body weight ($\pm$ S.E.M.) for each group was: fed, 221 ± 16 g; starved, 188 ± 19 g; non-ketotic diabetic, 216 ± 12 g; ketotic diabetic, 182 ± 6 g; DEHP-treated, 208 ± 9 g.

CPT purification

Mitochondria were prepared from livers of DEHP-treated rats by differential centrifugation (Hoppel & Tomec, 1972; Hoppel *et al.*, 1979). This isolation procedure discards light mitochondria, which contain microsomal enzyme activity (Hoppel & Tomec, 1972). The contamination of the heavy mitochondria, which are retained in this procedure, with other subcellular marker enzymes is minimal (Hoppel & Tomec, 1972). We have confirmed that peroxisomal contamination (based on catalase and on cyanide-insensitive palmitoyl-CoA-stimulated NAD⁺ reduction) of these preparations is less than 5%, and we have further found that carnitine octanoyltransferase activity is undetectable.

Mitochondrial suspensions were pooled and solubilized with 4% Tween/1 M-KCl/0.1 mM-phenylmethanesulphonyl fluoride/0.5 mM-dithiothreitol. Preliminary studies showed that Tween 20 addition did not adversely affect CPT activity. The CPT was purified as described previously (Brady & Brady, 1986). This procedure incorporates a gel-filtration step (Sephacryl S-300; Pharmacia, Piscataway, NJ, U.S.A.), as described by Clarke & Bieber (1981), an anion-exchange step (DEAE-Fractogel; EM Science, Gibbstown, NJ, U.S.A.), as described by Miyazawa *et al.* (1983), and chromatofocusing (Pharmacia) with a pH-gradient of 7–5. The procedure yields a single band on SDS/polyacrylamide-gel electrophoresis with M_r 68 000 (12.5%-acrylamide gel; Laemmli, 1970). Protein concentration of purified CPT was determined as described by Bradford (1976).

Determination of detergent effects

There have been suggestions that the use of detergent destroys one of the CPT proteins (Declercq *et al.*, 1985). Hoppel *et al.* (1986) have used sonicated mitochondrial fractions for the purification of a CPT activity with different properties from those observed when detergents are used. Thus mitochondria were isolated as described above and then suspended in 20 mM-potassium phosphate/1 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride buffer, pH 7.4. The mitochondria were sonicated as described previously (Hoppel, 1982; Brady & Brady, 1986) and centrifuged at 100 000 *g* for 1 h. The supernatant is termed 'sonicated supernatant'. The pellet from this preparation is referred to as 'sonicated pellet', and was resuspended in the phosphate/EDTA/phenylmethanesulphonyl fluoride buffer described above. CPT activity was assayed (80 μ M-palmitoyl-CoA, 8 mM-L-carnitine) in the presence of 0 (control) or 0.1 % of various detergents by using purified CPT, sonicated pellet and sonicated supernatant (Table 1). Digitonin, Zwittergent 3–16, Triton X-100 and reduced Triton all significantly decreased CPT activity. Octyl glucoside was strongly stimulatory. CHAPS [3-(3-cholamidopropyl)dimethylammonio-1-propanesulphonate], Lubrol PX and Tween 20 had little effect. Purified pigeon breast carnitine acetyltransferase was used as a control and was unaffected by detergent addition (results not shown). Both sonicated supernatant and sonicated pellet were then concentrated and dialysed before antibody precipitation and the Western blot studies, and were stored frozen before use. Samples of each were passed through a chromatofocusing column by using a pH 7–5 gradient, and elution profiles of CPT activity relative to pH were monitored. It is possible that all CPT activity in the pellet was not expressed, owing to incomplete dissolution of the membranes, but it should be noted all solubilized mitochondrial CPT is precipitated when the same preparation is not fractionated.

Antibody characterization

Although evidence exists that CPT activity is the product of a single protein (Clarke & Bieber, 1981; Miyazawa *et al.*, 1983), some debate still exists (Declercq *et al.*, 1985; Ramsay *et al.*, 1987). At the same time, measurement of turnover of the CPT protein and translation of the specific mRNA are dependent on the quality (lack of ambiguity) of the antibody probe. In the course of the characterizations of the antibody, evidence was provided for a single CPT protein.

Polyclonal antibody to purified rat hepatic CPT was raised in female BALB/c mice and used as crude defibrinated ascites fluid (Tung, 1983). We have presented data above supporting the use of Tween-20-solubilized mitochondria as a source of purified CPT. Ouchterlony plates were prepared as described previously (Ouchterlony, 1966). Purified rat liver CPT, sonicated pellet and sonicated supernatant yielded a single, continuous, precipitin line without spurs (Fig. 1, insert). Thus the antigenic determinants are the same for all preparations. Purified CPT, Tween-20-solubilized hepatic mitochondria, sonicated pellet (no detergent added) and sonicated supernatant (no detergent added) were diluted to a uniform CPT activity (0.1 A_{412} /min per ml, or approx. 0.35 μ g of CPT protein/ml). Graded amounts of antibody preparation were then added to a final volume

Fig. 1. Sensitivity of various CPT preparations to antibody

Purified CPT (●), sonicated supernatant (▲) and pellet (■) were prepared as described in the Experimental section. CPT activity was adjusted by dilution to yield an activity of $0.1 A_{412}$ unit/min per ml. Increasing amounts of antibody, as crude ascites fluid, were added to a final volume of 1 ml and incubated, as described in the Experimental section, at room temperature. The inset shows an Ouchterlony plate: C, purified CPT; S, sonicated supernatant; P, sonicated pellet.

of 1 ml in 20 mM-Tris/HCl/0.9% NaCl, pH 8, and incubated at 37 °C for 1 h. Protein A-agarose was added and incubation continued for an additional 1 h. The antibody-antigen-protein A complex was precipitated by centrifugation (12000 g) and the CPT activity of the supernatant assayed as described above. Non-specific effects were removed by using antibody generated to pigeon breast carnitine acetyltransferase as a control. These data are presented (Fig. 1) and demonstrate that all of these CPT preparations were equally sensitive to the antibody. Complete precipitation of the activity occurred with slightly less than 30 μl of the anti-CPT antibody preparation.

Various mitochondrial preparations were subjected to SDS/polyacrylamide-gel electrophoresis (12% acrylamide), followed by electrophoretic transfer in 25 mM-Tris/192 mM-glycine/20% (v/v) methanol, pH 8.3, to 0.2 μm nitrocellulose membranes at 500 mA overnight (Towbin *et al.*, 1979). Pre-stained protein standards were used for calibration (Diversified Biotech, Newton Centre, MA, U.S.A.). After blocking with 5% (w/v) bovine serum albumin in 20 mM-Tris/HCl/0.9% NaCl, pH 8.2, the nitrocellulose was exposed to primary antibody (50–200 μl/50 ml at 25 °C for 2 h). The antibody-antigen

Fig. 2. Western blots of various mitochondrial preparations

Samples of mitochondria (M), sonicated supernatant (S), sonicated pellet (P) and purified CPT (C) were prepared as described in the Experimental section and subjected to SDS-PAGE (10% acrylamide) prior to transfer to 0.2 μm nitrocellulose. The CPT was visualized using an alkaline phosphatase-linked second antibody system (Promega Biotec). The reference lines indicate the position of bovine serum albumin ($M_r = 67000$).

complex was detected after 1 h exposure of the nitrocellulose to anti-mouse IgG linked to alkaline phosphatase (Promega Biotec, Madison, WI, U.S.A.). The procedure was slightly modified such that all washes included 0.1% bovine serum albumin, and incubation with the second antibody also included 0.4% gelatin. This was found to be necessary to eliminate non-specific binding.

This Western-blot analysis indicated a single reactive species in Tween-20-solubilized mitochondria, in the sonicated supernatant, and in the sonicated pellet (Fig. 2). The electrophoretic mobility of the immunoreactive band corresponded to that of purified CPT. CPT activity in the sonicated supernatant and in the sonicated pellet were eluted from chromatofocusing at the same point, pH 6.0–5.5. Western blots again yielded only a single band of immunoreactive material in both of these preparations, with identical electrophoretic mobilities (results not shown).

These data provided evidence that the antigenic determinants were the same for all CPT preparations and that all CPT activity in each preparation was precipitated by the antibody. It can still be argued that two CPT proteins are synthesized with the same mobility on SDS/polyacrylamide-gel electrophoresis. To examine this further, using the translation system described below, we observed only a single CPT-immunoreactive band on SDS/polyacrylamide-gel electrophoresis with $M_r = 70490$. Again, this does not prove that there could not be two CPT proteins, but would require that they both be synthesized as a precursor with equivalent mobilities, and that they both be processed to produce final products of the same mobilities. In any case, it was concluded that the antibody was satisfactory for turnover and translation studies.

CPT turnover *in vivo*

Turnover studies were attempted only in fed and non-ketotic diabetic rats to allow a reasonable approximation of steady-state conditions. Fed (control) animals and

non-ketotic diabetic animals were injected intraperitoneally with 150 μ Ci of [2,3,4,5- 3 H]leucine. Rats were killed by decapitation 12, 24, 36, 48, 72, 96 and 120 h after [3 H]leucine injection. Blood was collected in heparin, and plasma was separated. Plasma glucose was measured by the hexokinase/glucose-phosphate dehydrogenase assay (Sigma Kit 16UV). All rats that had been judged diabetic by glucosuria showed plasma glucose when killed greater than 300 mg/dl (587 ± 25 mg/dl; mean $\pm$ S.E.M.). Two rats were excluded on this basis.

At each time point, the livers were removed into ice-cold MSM buffer, blotted, weighed and homogenized with a loose-fitting Teflon pestle. The procedure for mitochondrial isolation has been described in detail (Hoppel *et al.*, 1979). Homogenate and mitochondrial suspension volumes were measured, and a sample of each was frozen at -70°C for subsequent analysis.

These procedures were similar to those used by Ozasa *et al.* (1983), who examined CPT turnover in DEHP-treated rats. Although the use of the leucine label may overestimate the turnover rate, the use of [*guanido*- 14 C]-arginine is less desirable, because of the low occurrence of this amino acid in CPT (under 4 mol%, compared with almost 12 mol% for leucine) and because of the considerably lower specific radioactivity of commercially available [*guanido*- 14 C]arginine. The use of leucine did allow us to compare directly with the findings of Ozasa *et al.* (1983). To measure total hepatic mitochondrial protein turnover, mitochondria and an equal volume of 20% trichloroacetic acid were mixed to precipitate the protein. The pellet was washed twice with trichloroacetic acid, solubilized with Protosol and counted for radioactivity. Another sample of the mitochondria was diluted, and protein was measured by the biuret method (Gornall *et al.*, 1949). Total mitochondrial CPT was measured by the spectrophotometric 5,5'-dithiobis-(2-nitrobenzoate) method with 160 μ M-palmitoyl-CoA and 8 mM-L-carnitine (Bieber *et al.*, 1972), with the modification that mitochondrial samples after thawing were diluted in 0.5% Tween 20/5 mM-phosphate/1 mM-phenylmethanesulphonyl fluoride/0.5 mM-dithiothreitol, pH 7.4. The total volume was then made to 1 ml with water to give 2 mg of mitochondrial protein/ml. Thus the Triton X-100 was omitted from the dilution and assay, since we have found that Triton inhibits CPT activity. The palmitoyl-CoA hydrolysis by palmitoyl-CoA hydrolase was assayed without L-carnitine addition and was subtracted from the activity obtained in the presence of L-carnitine (this amounted to about 20% of the rate with L-carnitine in solubilized thawed mitochondria). Citrate synthase was measured as a mitochondrial marker in both homogenate and mitochondria (Srere, 1969). On the basis of citrate synthase activity, mg of mitochondria per g of liver and per liver were calculated. Mitochondrial CPT data were then expressed per mg of mitochondrial protein and as total μ g of hepatic CPT, calculated from the specific activity of the purified CPT at 25°C without added detergent (2.5 units/mg).

A standard curve of antibody addition versus CPT activity removed was developed. It was found that the activity precipitated per unit of antibody was the same for control and diabetic rats (results not shown). A portion of homogenate was solubilized with an equal volume of 2% Tween 20/0.5 mM-phenylmethane-

sulphonyl fluoride/0.5 mM-dithiothreitol, and a 5-fold excess of antibody was added to effect complete precipitation. Homogenate and antibody were allowed to react at room temperature for 2 h. After this period, Protein A-agarose beads were added and allowed to react for 1 h. The precipitate was pelleted by centrifugation (12000 *g* for 3 min) and was washed twice with 10 mM-Tris/150 mM-NaCl, pH 8.0, containing 0.05% Tween 20. The final pellet was dissolved in a small volume of Protosol and counted for radioactivity. The decay of radioactivity in CPT for both groups is summarized (Fig. 3). The k_d was calculated from this plot of the log of radioactivity (d.p.m.) versus time. The half-life was calculated as $k_d = 0.693/t_{1/2}$. The k_s (synthesis constant) was determined from $k_s = \frac{1}{2} Ck_d$, where C is the steady-state amount of CPT, in μ g per liver. In order to ensure that the diabetic animals were, in fact, in steady state, the body weight and plasma glucose were monitored. As noted above, during the 120 h after leucine injection, control and diabetic rats did not differ significantly in weight from the initial value. Further, plasma glucose did not change with the time after injection of [3 H]leucine.

mRNA translation rates

Livers were removed from rats receiving the various treatments (four to seven per treatment), and samples were homogenized directly in 4 M-guanidium isothiocyanate. RNA was isolated from each animal, and treated individually, by the chloroform/hot-phenol method. Polyadenylated RNA was purified by oligo-(dT)-cellulose chromatography (Maniatis *et al.*, 1982). The mRNA was detected by ethidium bromide staining after electrophoresis in 1% agarose containing formaldehyde (Maniatis *et al.*, 1982). There was no evidence of significant RNAase hydrolysis. Further, when cDNA was prepared for insertion into λ gt11, the size range of the inserts was generally large and consistent with minimal hydrolysis. The A_{260}/A_{280} was 1.8 or better. The polyadenylated RNA (mRNA) was translated, in triplicate, in a rabbit reticulocyte lysate system containing, in 50 μ l final volume, 50 units of RNasin (Promega Biotec), 140 mM-potassium acetate, 1 mM-magnesium acetate and 0.1 mCi of [2,3,4,5- 3 H]leucine/ml at 30°C . The reaction was diluted to 1 ml with 0.1 M-NaCl/1 mM-EDTA/1% Nonidet P-40/10 mM-Tris/HCl, pH 7.5, and excess antibody added. After a 2–3 h incubation, 20 μ l of Protein A-agarose was added and incubated for 1 h. The pellet was isolated by centrifugation (12000 *g* for 3 min) and washed three times before radioactivity counting. Suitable blanks, with Protein A-agarose plus goat serum, were subtracted for non-specific precipitation. The blanks amounted to 10–15% of total radioactivity (c.p.m.). The putative CPT was also translated by using mRNA from two rats each of the fed, non-ketotic diabetic and ketotic diabetic groups, and antibody-precipitated as above, followed by SDS/polyacrylamide-gel electrophoresis. When the zone corresponding to the CPT (based on SDS/polyacrylamide-gel electrophoresis as well as Western blots) was cut out and counted for radioactivity, values were comparable with those obtained with antibody precipitation alone. The mRNA translation was evaluated by Western-blot analysis of the CPT translation product, and the M_r of the product was 70490, which is in agreement with the previous report by Ozasa *et al.* (1983). The mRNA concentration and

incubation times were determined by preliminary trials, and were 60 μ g of mRNA/ml for 60 min at 30 °C unless noted. Total translation products (trichloroacetic acid-precipitable) were not different among the various treatment groups.

Data expression

Mitochondrial protein per g of liver was determined by using citrate synthase as a mitochondrial marker enzyme. Total citrate synthase activity of the homogenate was calculated by multiplying the specific activity (units/ml of homogenate) by the total volume and dividing by the weight (g) of liver homogenized. The mitochondrial citrate synthase activity per mg of mitochondrial protein was determined by direct measurement.

Total homogenate citrate synthase/g of liver was divided by mitochondrial citrate synthase/mg of mitochondrial protein to give the mg of mitochondrial protein per g of liver. The values obtained (60–100 mg/g) were similar to our previous results (Brady & Hoppel, 1983a,b).

Materials

CoA was purchased from Pharmacia-PL Biochemicals. Palmitoyl-CoA was synthesized as described by Seubert (1960) by using commercially available palmitoyl chloride (Sigma). Purified pigeon breast carnitine acetyltransferase, Triton X-100 and reduced Triton, Lubrol PX and octyl glucoside were purchased from Sigma. Digitonin was also purchased from Sigma, but recrystallized from hot ethanol before use. Zwittergent 3-16 and CHAPS were purchased from Calbiochem (LaJolla, CA, U.S.A.). Tween 20 was purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). L-Carnitine was a gift from Sigma-Tau, Rome, Italy. Nitrocellulose membranes (0.45 and 0.2 μ m) were purchased from Hoefer Scientific. Rabbit reticulocyte-lysate translation and processing kit (endonuclease-treated) were purchased from DuPont-New England Nuclear, Boston, MA, U.S.A., or from Promega Biotec. Protosol was purchased from DuPont-New England Nuclear. RNasin was purchased from Promega Biotec. The [2,3,4,5-³H]-

leucine (sp. radioactivity 110 Ci/mmol) was obtained from ICN Biochemicals (Irvine, CA, U.S.A.). Protein A was obtained from Pierce Chemicals (Rockford, IL, U.S.A.). Streptozotocin was kindly given by the Upjohn Co. (Kalamazoo, MI, U.S.A.). All other chemicals were reagent grade or better.

RESULTS AND DISCUSSION

Characteristics of CPT and anti-CPT antibody

In order to measure turnover of CPT and its relative translation rate, it was necessary to document the nature of the purified CPT used to generate antibody and the quality of the antibody produced. The question has been raised whether detergents might destroy one of the CPT activities, thus leading to the view of a single CPT protein. In our preliminary characterization, which is presented in some detail in the Experimental section, we find that various detergents do indeed alter the apparent velocity of the CPT reaction (Table 1). It is well known that the condition of assay of CPT will govern the apparent rate of reaction. The inclusion of lipids (Pande *et al.*, 1986; Woldegiorgis *et al.*, 1985), dimethyl sulphoxide (Brady & Brady, 1986), and detergents (Bremer *et al.*, 1985; Clarke & Bieber, 1981a; Miyazawa *et al.*, 1983) are known to alter the results obtained, and we have documented specific effects of various detergents on purified CPT, and the CPT activity of sonicated supernatant and pellet (prepared without detergent). Thus we believe that the CPT that we purified from detergent-solubilized mitochondria was the product of a single protein or of two proteins with identical electrophoretic mobilities.

The documentation of the antibody provides further insight into this question. The anti-CPT antibody precipitated all of the CPT activities in preparations (sonicated pellet and supernatant) which have been proposed to be enriched in CPT-B and CPT-A respectively (Hoppel, 1982). There was no evidence of differences in antibody reactivity in these preparations relative to that of the purified CPT, as evidenced by this

Table 1. CPT activities in the presence of various detergents

CPT activities (purified CPT, sonicated pellet and sonicated supernatant were prepared as described in the Experimental section) are presented as a fraction of the activity determined in the absence of detergent, with 80 μ M-palmitoyl-CoA and 8 mM-L-carnitine, by the spectrophotometric assay at 25 °C. All detergents were added to 0.1 %, except for octyl glucoside, which was added at 5 mM. Values are means $\pm$ S.D. with the numbers of independent determinations presented in parentheses.

Detergent	Activity		
	Purified CPT	Sonicated supernatant	Sonicated pellet
None	1.00 $\pm$ 0.06 (3)	1.00 $\pm$ 0.31 (8)	1.00 $\pm$ 0.10 (3)
Tween 20	1.19 $\pm$ 0.01 (3)	1.10 $\pm$ 0.17 (3)	1.46 $\pm$ 0.39 (2)
Triton X-100	0.58 $\pm$ 0.10 (5)	0.50 $\pm$ 0.31 (4)	0.69 $\pm$ 0.21 (3)
Reduced Triton	0.62 $\pm$ 0.21 (2)	0.57 $\pm$ 0.11 (2)	0.61 $\pm$ 0.23 (2)
Digitonin	0.51 $\pm$ 0.23 (2)	0.30 $\pm$ 0.32 (3)	0.43 $\pm$ 0.04 (4)
Lubrol PX	1.00 $\pm$ 0.11 (3)	1.18 $\pm$ 0.25 (3)	1.02 $\pm$ 0.19 (3)
Zwittergent 3-16	0.08 $\pm$ 0.01 (4)	0.19 $\pm$ 0.08 (3)	0.10 $\pm$ 0.04 (3)
Brij 96	0.83 $\pm$ 0.07 (3)	0.92 $\pm$ 0.12 (2)	0.87 $\pm$ 0.17 (3)
Octyl glucoside	1.70 $\pm$ 0.26 (5)	2.19 $\pm$ 0.65 (4)	2.01 $\pm$ 0.30 (3)

Table 2. CPT total hepatic protein and mitochondrial protein values and turnover kinetic parameters in control and streptozotocin-diabetic rats

Streptozotocin-diabetic rats (non-ketotic) were produced by injecting rats with 55 mg of streptozotocin/kg body wt. The rats were maintained for 1 week before injection with [2,3,4,5-³H]leucine. Control animals were maintained for the same period without streptozotocin injection. After the [³H]leucine injection, rats were killed (three per time point) at 12, 24, 36, 48, 72, 96 and 120 h, and the liver was removed and processed as described in the Experimental section. The data at each time point were used to estimate parameters. In addition, liver protein, mitochondrial protein and CPT activity were determined as described in the Experimental section. Values are expressed as means $\pm$ S.E.M. for 20 (control) or 21 (diabetic) animals. Statistical significance ($P < 0.05$) is indicated by an asterisk (*).

	Control	Diabetic	Diabetic/control
CPT			
(nmol/min per mg of mitochondrial protein)	37.1 $\pm$ 1.1	34.3 $\pm$ 1.8	0.92
(μ g/liver)	724 $\pm$ 46	990 $\pm$ 57*	1.37
k_d (day ⁻¹)	0.352	0.361	1.03
$t_{1/2}$ (days)	2.0	1.9	0.95
k_s (μ g/day)	255	357	1.40
Mitochondrial protein			
(mg/g of liver)	62.4 $\pm$ 3.7	94.1 $\pm$ 5.3*	1.51
(mg/liver)	414 $\pm$ 26	700 $\pm$ 44*	1.69
k_d (day ⁻¹)	0.20	0.18	0.90
$t_{1/2}$ (days)	3.5	3.9	1.12
k_s (mg/day)	82.8	126.0	1.52

precipitation (Fig. 1) and by the lack of spurs in the Ouchterlony plates (Fig. 1, insert). Further, the total solubilized mitochondria, sonicated supernatant and sonicated pellet exhibited a single immunoreactive band on Western blots, again corresponding to the purified CPT. The argument remains for multiple CPT proteins with equivalent electrophoretic mobilities. However, only a single immunoreactive precursor was observed on translation of hepatic mRNA, with M_r 70490. Ozasa *et al.* (1983) have previously reported this precursor; however, it is not absolutely certain that their antibody reacted to all CPT activity. Although it may still be argued that there are two CPT proteins, this would now require the synthesis of precursors with identical electrophoretic mobilities, which are cleaved to products, again with equivalent mobilities. We cannot rule this out, but consider it unlikely. In any case, we have documented that the antibody is of adequate quality to use as a tool for the turnover and translation studies discussed below.

CPT turnover in non-ketotic diabetes

The data for CPT turnover are presented in Table 2. Total CPT activity, expressed as nmol/min per mg of mitochondrial protein, was not different in control and mild diabetic rats. These data are consistent with those of Saggerson & Carpenter (1981), who found that hepatic CPT-A (outer) activity and malonyl-CoA-sensitivity were the same in control and hyperglycaemic non-ketotic streptozotocin-diabetic rats when the same protocol as in the present study was used to produce non-ketotic diabetes. In contrast, Cook *et al.* (1984) injected 150 mg of streptozotocin/kg and used the animals after 48 h because ketosis was maximal at this time. We have routinely maintained the animals for at least 1 week after either the low dose (55 mg/kg) or the high dose (110 mg/kg; Brady *et al.*, 1985) to decrease the possibility that any effects seen may be related to early toxicity of streptozotocin to the liver or kidney (Wieland, 1968; Brady

et al., 1982, 1985). Contrary to the present results, Harano *et al.* (1972a) found that non-ketotic hyperglycaemic rats had increased hepatic mitochondrial CPT activity (both CPT-A activity and total CPT activity). Those investigators used 60–70 mg of alloxan/kg body wt. and killed the rats 1 week after injection. It is unclear why CPT activity in these alloxan-diabetic animals, relative to their fed control, was higher than the CPT activity of our streptozotocin-diabetic animals, relative to our fed control. In our subsequent studies to examine translation rates, we observed higher, but again not significant, increases in CPT activity in the non-ketotic diabetic animals. In the present study, we also report higher total mitochondrial CPT than did Harano *et al.* (1972a) for both controls and hyperglycaemic non-ketotic diabetic rat liver (37 and 34 nmol/min per mg of mitochondrial protein in the present study, versus 10.9 and 16.3 in their study). DiMarco & Hoppel (1975) have used the reverse CPT assay (palmitoyl-CoA + carnitine formation) to measure total hepatic mitochondrial CPT, and found that controls and ketotic diabetics had the same CPT activity. Our previous experiments (Brady *et al.*, 1985) and those of others (Harano *et al.*, 1972a; Nosadini *et al.*, 1979) indicated increased CPT activity of both CPT-A and CPT-B in ketotic diabetic rats. However, it should also be noted that CPT activity measured in the forward direction (palmitoylcarnitine formation) may be affected by the same perturbant differently from CPT activity measured in the reverse direction (palmitoyl-CoA formation) (Pande *et al.*, 1986).

When CPT protein per total liver was calculated, the mildly diabetic animals exhibited significantly greater amounts of CPT protein. Thus, although the increase in CPT activity per mg of mitochondrial protein in non-ketotic diabetic rats was not as dramatic as is generally observed for the ketotic diabetic rat, there is clear evidence that the total hepatic CPT has increased in this milder non-ketotic diabetic model, owing to the increased mitochondrial protein per g of liver and per liver (Table

Fig. 3. Decay curves of the radioactivity in CPT

Each value is the mean for three rats. Diabetic rats were prepared as described in Table 1 and in the Experimental section. CPT was isolated from liver homogenate by immunoprecipitation as described in the Experimental section. Radioactivity (d.p.m.) as a fraction of the initial value (derived from extrapolation to initial time by linear regression) is plotted semi-logarithmically versus time (h). ●, Control rats; ■, diabetic rats. Lines were derived by least-squares fit.

2). Harano *et al.* (1972b) have shown increased hepatic mitochondrial protein with experimental diabetes, which is associated with increases in mitochondrial size without increased mitochondrial number. Therefore, increases in CPT activity with diabetes must reflect increases in CPT protein per mitochondrion rather than increased mitochondrial numbers with constant CPT protein. Thus, per animal, or per 100 g body wt., hepatic CPT was increased in non-ketotic diabetes.

The CPT degradation rate (k_d) and the half-life ($t_{1/2}$) were essentially the same for control and non-ketotic diabetic rats (Table 2 and Fig. 3). However, k_s was 40% greater in the mild-diabetic state. It might be expected that turnover parameters would be more dramatically altered in ketotic diabetic animals; however, turnover *in vivo* cannot be estimated under the conditions of this experiment in ketotic diabetic rats, because of the lack of steady-state conditions. Ozasa *et al.* (1983) have pre-

viously reported a $t_{1/2}$ for hepatic CPT of 6.5 days (156 h), compared with the 1.9–2.0 days in the present study. It is unclear why we observe some difference in our estimate, since, to the extent possible, conditions of control animals were identical.

In the present study, CPT appears to turn over slightly more rapidly than the average mitochondrial protein, which was estimated at 3.5–3.9 days in the present study. Weinberg & Utter (1979) have previously reported a $t_{1/2}$ for rat liver mitochondrial protein of 3.8 days.

CPT translation

We next examined mRNA translation activity for CPT in various physiological states. These data, with the corresponding CPT activities, are presented in Table 3. As mentioned above, CPT activity per mg of mitochondrial protein was not significantly increased with non-ketotic diabetes, but was increased approx. 2-fold by 48 h starvation, and by ketotic diabetes, and about 3.5-fold by DEHP treatment. Expressed as units/liver, the non-ketotic diabetic animals exhibited approximately the same small increase as in the study *in vivo* (37% versus 23% increase). A similar pattern was observed when activity was expressed per 100 g body wt. The accompanying CPT-mRNA translation activity showed a similar pattern: that is, non-ketotic diabetic rats had translation rates approx. 2-fold greater than fed controls (somewhat higher than the difference in k_s). The starved, ketotic diabetic and DEHP-treated rats had translation rates approx. 4-fold greater than the fed control. Thus, although all treatments are in qualitative agreement with the observed CPT activities, quantitatively the translation rates are somewhat higher than might be expected. Ozasa *et al.* (1983) have reported even greater disparity between control and DEHP-treated rats (5.9-fold increase in turnover; 28-fold increase in translation). It is unclear why they report such a large disparity compared with the values reported here. However, we used polyadenylated RNA, rather than their total cellular RNA. Total RNA is known to be more variable than mRNA in translation. It should also be noted that incorporation of radioactivity into immunoprecipitable CPT was very low (essentially immeasurable relative to total radioactivity) in their system. Our own system allowed measurable incorporation of label into CPT accounting for 0.037% of trichloroacetic acid-precipitable label incorporation in control rats. Since it is clear that CPT is synthesized as a larger precursor, processing may be a limiting factor. Membrane insertion and cleavage of this putative leader sequence will need to be examined in more detail. However, it is possible that the rate of processing of the precursor is limiting, and may be the reason why the ratios of treated/fed-rat mRNA-translation activities exceed the ratios of CPT activity.

A number of mechanisms have been proposed for the regulation of CPT activity. These include the synthesis/degradation process, changes in malonyl-CoA-sensitivity, phosphorylation/dephosphorylation and changes in the characteristics of the mitochondrial membrane. The data presented here indicate that long-term regulation is probably brought about by changes in the rate of synthesis of the CPT, which, in turn, is the product of increased mRNA. However, in spite of its regulatory role, CPT activity is increased only up to 3.5-fold in the present studies. Generally, increases are no more than 2-fold in the more physiological states, such as starvation

Table 3. CPT activities and mRNA translation rates in liver of fed, starved, diabetic and DEHP-treated rats

Rats were treated as described in the Experimental section. After they were killed a sample of liver was used for mitochondrial isolation and CPT activity determination. The remainder of the liver was used for mRNA isolation and translation. CPT activities are expressed as munits/mg of mitochondrial protein (nmol/min per mg of protein with 160 μ M-palmitoyl-CoA and 8 mM-L-carnitine) and as units/100 g body wt. Translation activity is expressed as [3 H]leucine (d.p.m.) incorporated in a 60 min incubation at 30 °C with 60 μ g of mRNA, and as the ratio of incorporation to that in the fed control. The incorporation (d.p.m.) was determined by antibody precipitation of the CPT as described in the Experimental section. Each value is the mean $\pm$ S.E.M. for four to seven animals (number is indicated in parentheses). Differences between means were tested by using Tukey's omega procedure. Within a column, different letter superscripts indicate significant ($P < 0.05$) differences.

Treatment	Body wt. (g)	Liver wt. (g)	CPT					Translation activity		
			(munits/ mg)	Treatment/ fed	(units/ liver)	Treatment/ fed	(units/100 g body wt.)	Treatment/ fed	(d.p.m.)	(Treatment/ fed)
Fed	222 ± 27 (7)	10.6 ± 1.6 (7)	39.4 ± 3.4 ^a (7)	1.00 ± 0.14 ^a (7)	43.5 ± 3.3 ^a (7)	1.00 ± 0.13 ^a (7)	19.6 ± 3.8 ^a (7)	1.00 ± 0.20 ^a (7)	448 ± 27 ^a (7)	1.00 ± 0.06 ^a (7)
48 h-starved	188 ± 33 (4)	7.2 ± 2.1 (4)	76.1 ± 5.4 ^b (4)	1.93 ± 0.13 ^b (4)	77.6 ± 9.8 ^b (4)	1.78 ± 0.11 ^b (4)	41.3 ± 3.4 ^b (4)	2.11 ± 0.18 ^b (4)	1316 ± 150 ^b (5)	2.94 ± 0.34 ^b (5)
Non-ketotic diabetic	216 ± 21 (7)	11.0 ± 1.8 (7)	52.8 ± 4.0 ^{ab} (7)	1.34 ± 0.15 ^{ab} (7)	53.3 ± 9.2 ^{ab} (7)	1.23 ± 0.15 ^a (7)	24.7 ± 3.1 ^{ab} (7)	1.26 ± 0.16 ^{ab} (7)	1024 ± 26 ^b (6)	2.29 ± 0.05 ^b (6)
Ketotic diabetic	182 ± 31 (4)	8.8 ± 1.7 (4)	78.6 ± 4.3 ^b (4)	1.99 ± 0.10 ^b (4)	79.4 ± 10.1 ^b (4)	1.84 ± 0.13 ^b (4)	43.6 ± 3.4 ^b (4)	2.22 ± 0.19 ^b (4)	1467 ± 242 ^b (6)	3.28 ± 0.54 ^{bc} (6)
DEHP- treated	208 ± 17 (4)	15.0 ± 0.9 (4)	137.2 ± 11.1 ^c (4)	3.48 ± 0.27 ^c (4)	137.7 ± 31.6 ^c (4)	3.17 ± 0.28 ^c (4)	66.2 ± 5.4 ^c (4)	3.38 ± 0.28 ^c (4)	2257 ± 170 ^c (4)	5.04 ± 0.38 ^c (4)

and ketotic diabetes. Thus changes in malonyl-CoA-sensitivity, phosphorylation and membrane alterations may be of considerable importance in the short-term regulation of CPT.

Note added in proof (received 20 July 1987)

In the course of these studies, we have discovered that the Pierce BCA protein assay gives more reproducible results than the Bradford (1976) protein assay when the CPT samples contain detergents.

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