Activity of branched-chain 2-oxo acid dehydrogenase complex in rat liver mitochondria and in rat liver

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Four mitochondrial marker enzymes were used to show that: (1) high-protein (24 %) diet increased the rat liver concentration and content of total branched-chain 2-oxo acid dehydrogenase complex (BCDC) by 31 % by increasing mitochondrial specific activity of BCDC; (2) starvation increased the liver concentration of BCDC by 25 % by decreasing liver weight; the liver content of mitochondria and the mitochondrial specific activity of BCDC were unchanged; (3) protein-free diet decreased rat liver BCDC concentration and content by 20 %, by decreasing the liver concentration and content of mitochondria. Protein-free diet increased liver mitochondrial specific activities of L-glutamate, 2-oxoglutarate and NAD-isocitrate dehydrogenases. The validity of a mitochondrial method for the determination of the liver concentration of BCDC and the percentage in the active form *in vivo* is confirmed, and improvements are described. The experimental basis of criticisms of its use in this regard by Zhang, Paxton, Goodwin, Shimomura & Harris [(1987) *Biochem. J.* **246**, 625–631] was not confirmed. The finding by Harris, Powell, Paxton, Gillim & Nagae [(1985) *Arch. Biochem. Biophys.* **243**, 542–555], that starvation has no effect on the percentage of BCDC in the active form in rat liver, is confirmed.

INTRODUCTION

Mitochondrial branched-chain 2-oxo acid dehydrogenase complex (BCDC) in animal tissues is inactivated by phosphorylation (BCD kinase) and re-activated by dephosphorylation (BCD phosphatase) [1–4]. In the rat, liver is the major site of active BCDC, and of its regulation by reversible phosphorylation in response to dietary protein content [5–8]. Low-protein diets decrease the percentage of BCDC in the active form in liver [5–8] and the liver concentration of total BCDC (sum of active and inactive forms) [7,8]. Starvation increases liver concentration of total BCDC [7,8] and has been variously reported to have no effect on percentage of active BCDC [7,8] or to decrease it [5,6]. Because BCDC is mitochondrial, its concentration in liver depends on the mitochondrial specific activity of total BCDC and the liver concentration of mitochondria. The liver content, which determines the hepatic contribution to whole-body BCDC, is dependent also on liver weight. These parameters are essential to an understanding of the regulation of liver contents of BCDC (total and active form), and their measurement is described here.

BCDC concentrations in rat liver extracts are too low for spectrophotometric assay of the holo-complex reaction by NAD⁺ reduction. Assays of BCDC in liver extracts have utilized ¹⁴CO₂ release from [¹⁴C]ketovaline or [¹⁴C]ketoleucine and a rat liver cytosolic phosphoprotein phosphatase to effect conversion of inactive BCDC into active BCDC [7,8]. The spectrophotometric assay has been used in our laboratory to assay BCDC in extracts of rat liver mitochondria; total BCDC was obtained after conversion of inactive BCDC into active

BCDC in intact but uncoupled mitochondria by endogenous BCD phosphatase [5,6], and the active form of BCDC *in vivo* was obtained by isolating mitochondria in the presence of inhibitors of BCD kinase (ketoleucine or dichloroacetate) and BCD phosphatase (NaF) [5,6]. To compare BCDC activities in different preparations of mitochondria, citrate synthase activity was used as an index of mitochondrial recovery and purity. Zhang *et al.* [9] have questioned the validity of these methods. They find that NaF plus ketoleucine may lower the specific activities of citrate synthase and BCDC in rat liver mitochondria, and suggest that citrate synthase may not be a reliable marker of mitochondrial purity and recovery. We have been unable to confirm their findings, and because these results are important in the interpretation of mitochondrial specific activity of BCDC they are given here. We have also defined the relationship between citrate synthase and total BCDC activities and compared the use of citrate synthase, GluDH, 2-ogDH and NAD-ICDH in the measurement of liver mitochondrial concentration and content.

EXPERIMENTAL

Materials and rats

Sources of chemicals, diets and rats are in refs [5,6,10], except leupeptin (synthetic), rotenone and sodium *threo*-D,L-isocitrate, which were from Sigma Chemical Co., Poole, Dorset, U.K. Rats were routinely fed on 17 % protein chow diet [5]. Other diets (24 % protein chow and 0 % casein, from Special Diet Services, Witham, Essex, U.K.) were given for 14 and 10 days respectively.

Abbreviations used: BCD(C), branched-chain 2-oxo acid dehydrogenase (complex); GluDH, L-glutamate dehydrogenase; 2-ogDH, 2-oxoglutarate dehydrogenase complex; NAD-ICDH, NAD-isocitrate dehydrogenase; ketoleucine, 4-methyl-2-oxopentanoate; ketovaline, 3-methyl-2-oxobutyrates; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Rat liver mitochondria

Mitochondria were isolated in sucrose medium [5]; for assay of the active form of BCDC *in vivo*, sucrose medium contained 100 mM-NaF and 1 mM-sodium dichloroacetate (re-adjusted to pH 7.4). Unless stated, the first mitochondrial pellet was washed twice by resuspension and sedimentation. For assay of total BCDC, mitochondria (7.5 mg of protein/ml) were incubated at 30 °C in KCl medium containing 10 μ M-CCCP, with or without 5 mM-ketoleucine or 1 mM-dichloroacetate, for 30–60 min (17%- or 24%-protein diets or starvation) or 120 min (0%-casein diet) [6]. Mitochondria for assay of active form of BCDC *in vivo* were dispensed into KCl medium but not incubated. Mitochondria were pelleted by centrifugation (60 s; Eppendorff 5414), the supernatant was aspirated, and pellets were frozen in liquid N₂. Extracts (250 μ l; 30 mg of protein/ml [6]) were assayed for BCDC, citrate synthase and protein (Table 1, Fig. 1), or GluDH, 2-ogDH, NAD-ICDH, citrate synthase and protein (Table 2).

Rat liver extracts

Liver samples (approx. 2 g) were rapidly homogenized at 0 °C (Polytron homogenizer, position 4, four strokes) in 10 ml of 50 mM-potassium phosphate/5 mM-dithiothreitol/5 mM-EGTA/1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride/0.3 mM-tosyl-lysyl chloromethane/10 μ M-leupeptin, pH 7.5. After addition of 11.5 ml of the same medium supplemented with 5% (v/v) Triton X-100 and 4% (v/v) ox serum and mixing (two strokes, Polytron), the extract was frozen (liquid N₂) and thawed three times and 1 ml samples were clarified by centrifugation for 30 s.

Assays

BCDC was assayed by monitoring the rate of NAD⁺ reduction at 340 nm [11]. Citrate synthase was assayed as in ref. [12].

GluDH, 2-ogDH and NAD-ICDH were assayed spectrophotometrically in (respectively) 0.5, 10 or 40 μ l of liver or mitochondrial extracts by monitoring the rate of NADH oxidation (GluDH) or NAD⁺ reduction (2-ogDH, NAD-ICDH). The methods were as given in refs. [13,14], except that 30 mM-phosphate buffers were used containing rotenone (2 μ g/ml) and KCN (1 mM) to inhibit NADH oxidases. Near- V_{max} conditions were achieved with 1.5–2 mM-ADP, 2-oxoglutarate at 10 mM (GluDH) or 2 mM (2-ogDH) and 2 mM-DL-isocitrate. Blank rates (as percentages of the rates in the presence of these substrates) were: mitochondrial extracts < 2 (all assays); liver extracts 5.6 (GluDH), 3.3 (2-ogDH), 37 (NAD-ICDH). In all assays NADH oxidase was undetectable; rates were strictly proportional to extract concentration, and progress curves were linear for at least 2 min (longest period tested). 2-ogDH could not be detected in extracts of freshly prepared mitochondria, and activities of NAD-ICDH were very variable. Satisfactory assays were achieved after depleting mitochondria of endogenous substrates by incubation for 30 min at 30 °C in KCl medium containing 10 μ M-CCCP; prolonging the time of incubation to 120 min had no further effect on activities. Such mitochondria were used for all assays in Table 2. Incubation with CCCP had no effect on mitochondrial specific activities of GluDH or citrate synthase (results not shown). Protein was assayed against serum albumin standards [15].

Fig. 1. Stability of citrate synthase and of branched-chain dehydrogenase complex (active form) in rat liver mitochondria

Mitochondria were prepared in sucrose medium (three washes) from livers of two rats (chow diet; 17% protein) and equal samples were incubated at 0 °C for 3 h in sucrose medium alone (○), or containing 100 mM-NaF plus 1 mM-dichloroacetate (●), or 100 mM-NaF plus 5 mM-ketoleucine (▲). Duplicate samples (7.5 mg of protein) were taken at 0 (sucrose medium only), 30, 90 and 180 min, and after sedimentation the pellets were extracted (250 μ l volume) and extracts assayed in duplicate for citrate synthase, BCDC (active form) and protein. Total complex was assayed in extracts prepared from mitochondria incubated for 30 min at 30 °C in KCl medium containing 10 μ M-CCCP. Points are means of 16 assays on four mitochondrial preparations, and are shown with s.e.m. bars. There were no significant differences between any points in respect of activities of citrate synthase or BCDC (active form). Overall means $\pm$ s.e.m. for four preparations (eight livers) were: citrate synthase, 217 ± 2.1 munits/mg of protein; total BCDC, 11.1 ± 1 munits/mg of protein; BCDC (active form), 9.2 ± 0.15 munits/mg of protein ($81 \pm 0.7\%$ in the active form).

RESULTS AND DISCUSSION

Stability of BCDC and citrate synthase activities in rat liver mitochondria

Fig. 1 shows combined results for four experiments in which rat liver mitochondria prepared in sucrose medium were incubated for 3 h at 0 °C in sucrose medium with or without 100 mM-NaF/5 mM-ketoleucine or 100 mM-NaF/1 mM-dichloroacetate. The mitochondrial specific activities of BCDC or citrate synthase remained unchanged over the 3 h period under all three

Table 1. Branched-chain dehydrogenase complex in rat liver

BCDC (total) was assayed spectrophotometrically with ketoleucine as substrate in extracts of mitochondria isolated in sucrose medium and incubated in KCl medium containing 10 μ M-CCCP or 10 μ M-CCCP/5 mM-ketoleucine or 10 μ M-CCCP/1 mM-dichloroacetate. The active form *in vivo* was assayed in extracts of mitochondria isolated in sucrose medium containing either 100 mM-NaF/5 mM-ketoleucine (reference codes A, C) or 100 mM-NaF/1 mM-dichloroacetate (reference code B). Total activities were adjusted to a mitochondrial citrate synthase activity of 250 munits/mg of protein (see the text). Citrate synthase (line 5) was assayed in mitochondria isolated in sucrose medium. Reference codes: A, Patston *et al.* [5,6]; B, the present work; C, Patston *et al.* [6]; D, Beggs *et al.* [20]. Results are means $\pm$ S.E.M. for the numbers of mitochondrial preparations shown in parentheses. * $P < 0.01$, † $P < 0.02$, ‡ $P < 0.05$ for difference from 17%-protein, fed; § $P < 0.01$, || $P < 0.05$ for difference from 17%-protein, starved; ¶ $P < 0.05$ for difference from 24%-protein, fed. For other differences $P < 0.05$.

Diet...	17% protein		24% protein		0% protein
	Fed A, B, D	Starved (48 h) B	Fed B	Starved (48 h) B	Fed B, C, D
BCDC (adjusted total):					
(munits/mg of mitochondrial protein)	13.4 $\pm$ 0.39¶ (40)	13.6 $\pm$ 0.88 (8)	17.8 $\pm$ 0.7* (4)	17.1 $\pm$ 0.44§ (4)	13.8 $\pm$ 0.47¶ (15)
(munits/g fresh wt. of liver)	718 $\pm$ 21¶§ (40)	898 $\pm$ 54* (8)	940 $\pm$ 48* (4)	1115 $\pm$ 55¶ (15)	574 $\pm$ 26*¶ (15)
(units/liver)	8.8 $\pm$ 0.3¶ (40)	7.9 $\pm$ 0.6 (8)	11.3 $\pm$ 0.6* (4)	9.9 $\pm$ 0.5 (15)	6.6 $\pm$ 0.4*¶ (15)
BCDC (% active form)	49 $\pm$ 2.2¶ (40)	50 $\pm$ 4.4 (8)	90 $\pm$ 5* (4)	81 $\pm$ 7§ (15)	3.5 $\pm$ 0.35*¶ (15)
Citrate synthase					
(munits/mg of mitochondrial protein)	206 $\pm$ 7.2 (4)	209 $\pm$ 7.4 (4)	195 $\pm$ 3.7 (4)	200 $\pm$ 4.8 (4)	198 $\pm$ 6.2 (6)
(units/g fresh wt. of liver)	13.4 $\pm$ 0.2§ (4)	16.5 $\pm$ 0.4* (4)	13.2 $\pm$ 0.3 (4)	16.3 $\pm$ 0.4¶ (4)	10.4 $\pm$ 0.7*¶ (4)
(units/liver)	163 $\pm$ 4 (4)	146 $\pm$ 10 (4)	157 $\pm$ 12 (4)	145 $\pm$ 11 (4)	119 $\pm$ 10* (4)
Liver wt. (g)	12.1 $\pm$ 0.2§ (4)	8.9 $\pm$ 0.5* (4)	12.0 $\pm$ 1 (4)	9.0 $\pm$ 5¶ (4)	11.4 $\pm$ 0.2‡ (4)

conditions of incubation. There was no evidence for the 45% decrease in BCDC specific activity or the (up to) 8% release of citrate synthase to the medium as observed by Zhang *et al.* [9]. The only obvious experimental differences are that we used 5 mM-Tris, as in the original method of Patston *et al.* [5], and rats fed on 17%-protein diet, whereas Zhang *et al.* [9] used sucrose medium containing 20 mM-Hepes and rats fed on 24%-protein diets. The proportion of BCDC in the active form in mitochondria isolated in sucrose medium (81%; Fig. 1) was comparable with the 73% reported in [5]. The value is higher than the 49% obtained with mitochondria isolated in the presence of inhibitors of BCD kinase and phosphatase (Table 1), because of re-activation of BCDC in their absence.

The citrate synthase specificity activity of mitochondria prepared from 67 livers of rats fed on diets containing 17% protein was not influenced by addition to sucrose medium of 100 mM-NaF/5 mM-ketoleucine or 100 mM-NaF/1 mM-dichloroacetate. In these experiments single livers were divided between the two media. The values, in munits of citrate synthase/mg of protein, means $\pm$ S.E.M. (n), were: sucrose 124 $\pm$ 3.46 (17), sucrose/100 mM-NaF/5 mM-ketoleucine 127 $\pm$ 2.41 (17); and sucrose 164 $\pm$ 6.31 (50), sucrose/100 mM-NaF/1 mM-dichloroacetate 159 $\pm$ 6.18 (50). There was no evidence for the 36% decrease in citrate synthase specific activity induced by 100 mM-NaF/5 mM-ketoleucine in the experiments of Zhang *et al.* [9]. In applying the method to the assay of pyruvate dehydrogenase complex in liver, Denyer [16] observed no

difference in citrate synthase specific activities between eight mitochondrial preparations made in sucrose medium with or without 50 mM-NaF/5 mM-sodium pyruvate (ratio of activities 1.0 $\pm$ 0.03).

Zhang *et al.* [9] found that NaF/ketoleucine had a negligible effect (2.7%) on the release of citrate synthase from liver mitochondria kept at 0°C for 3 h in sucrose medium. This would suggest that the 36% decrease in citrate synthase specific activity which they found in mitochondria isolated in the presence of NaF/ketoleucine was not due to loss of citrate synthase induced by their presence. A more likely explanation is that the mitochondria isolated in the presence of NaF/ketoleucine were less pure. This might also explain their apparently higher water content [9]; it is known that contamination with other vesicular membrane fractions can increase the apparent water content of rat liver mitochondrial preparations [17].

Relationship between citrate synthase and total branched-chain complex activities in rat liver mitochondria

In our laboratory citrate synthase has been used routinely as an index of purity in comparing total BCDC specific activities in different liver mitochondrial preparations. Its activity is high, assay is rapid, blank rates are low and its specific activity is not changed by incubation of mitochondria to effect conversion of inactive BCDC into active BCDC.

The citrate synthase specific activities of mitochondria

prepared in sucrose medium from livers of 44 rats fed on 17%-protein diet varied over the range 61–250 munits/mg of protein; total BCDC activities ranged from 4.9 to 15.2 munits/mg of protein. The variation depended on procedures which influence the degree of purification (i.e. number of washes, time taken over resuspension). It was important to define the relationship between the two enzyme activities, because of its bearing on the calculation of mitochondrial specific activity of total BCDC. By least-squares linear-regression analysis, over the range of citrate synthase activities given, the relationship was linear and the slope (mean $\pm$ S.E.M.) was 36.74 ± 4.6 munits of BCDC/unit of citrate synthase ($P < 0.001$) ($r = 0.78$, $P < 0.001$).

This experimentally determined relationship has been used to calculate total BCDC in rat liver mitochondria at a citrate synthase specific activity of 250 munits/mg of protein by the equation:

$$\text{BCDC (citrate synthase = 250 munits/mg)} = \text{BCDC (citrate synthase = } x \text{ munits/mg)} + 0.03674(250 - x)$$

A value of 250 munits/mg for citrate synthase specific activity was chosen because it corresponds to the highest value observed and yields estimates (in rats fed on 17%- or 24%-protein diets) of approx. 55 mg of mitochondrial protein/g fresh wt. of liver. These correspond closely to the lowest of the published estimates of liver mitochondrial concentration (52.5 mg of protein/g of liver) obtained by Leighton *et al.* [18] from activities of marker enzymes and electron microscopy. There was close agreement in our own study between estimates obtained with citrate synthase, GluDH and 2-ogDH (see Table 2 and the next section).

No corrections were required for the percentage of BCDC in the active form, as there were no systematic differences in citrate synthase specific activities between mitochondria used to estimate total BCDC or active form of BCDC *in vivo* (see the preceding section).

Effect of dietary protein and of starvation on mitochondrial specific activity of BCDC, liver concentration and content of BCDC and liver concentration of mitochondria

Table 1, lines 1–4, shows BCDC activities obtained in this laboratory by the mitochondrial method (spectrophotometric assay; ketoleucine as substrate). In comparing these values, in what follows, with those obtained by others with radioactive assays employing ketovaline, the latter have been divided by 1.3 to correct for the higher $V_{\max}$ with ketovaline [19].

The specific activities of total BCDC in rat liver mitochondria adjusted to a mitochondrial citrate synthase specific activity of 250 munits/mg of protein are shown in Table 1, line 1. The value for 17%-protein diets is an overall mean (13.4 ± 0.39) derived from four studies; the individual adjusted means $\pm$ S.E.M. (source reference in brackets) were 13.3 ± 0.4 [5], 13.9 ± 0.3 [6], 13.2 ± 0.8 [20] and 12.3 ± 1.1 (new results). For 0%-casein diet the overall mean (13.8 ± 0.47) was derived from two studies; the individual adjusted means $\pm$ S.E.M. were 13.7 ± 0.29 [6] and 13.9 ± 0.9 [20].

The specific activity of total BCDC in liver mitochondria of rats fed on 17%-protein or 0%-casein diets did not differ significantly (Table 1, line 1, columns 2 and 6), but it was increased by 32% when rats were fed on 24%-protein diet (Table 1, line 1, columns, 2, 4 and 6).

This effect of higher protein diet was also apparent when non-adjusted values of total BCDC were compared in mitochondria matched for citrate synthase specific activities (means 197 and 199, range 187–209 munits/mg of protein). The corresponding specific activities for total BCDC, mean $\pm$ S.E.M. (n), were 12.2 ± 0.49 (10) and 15.4 ± 0.41 (8) ($P < 0.001$) for rats fed on 17%- and 24%-protein diets respectively. This would suggest that the mitochondrial concentration of BCDC may be increased when the protein content of diet is increased from 17 to 24%. Miller *et al.* [21] observed no significant effect of dietary protein (6–50% casein) on the specific activity of rat liver mitochondrial total BCDH. However, the relatively large S.E.M. values in their experiments may have obscured this difference.

Starvation (48 h) on either diet had no effect on mitochondrial specific activity of total BCDC (Table 1, line 1, columns 2–5). The adjusted values for 24%-protein diet (fed or starved) do not differ significantly from those reported by Zhang *et al.* [9] for a citrate synthase specific activity of 220–250 munits/mg of protein.

The specific activities of total BCDC in rat liver (Table 1, line 2) were computed from the specific activities of whole-liver citrate synthase (line 6) and the values for BCDC in line 1. Total liver contents are computed in line 3 from liver weights given in line 8. For measurement of liver weights and citrate synthase, rats from a single batch and of comparable weight were assigned in groups of four to the diets shown. The computed liver concentration and content of total BCDC was increased by approx. 30% when the protein content of the diet was increased from 17 to 24%. Liver weight was unchanged, i.e. 24%-protein diet increased the liver content of total BCDC. Starvation increased the computed concentration of total BCDC in rat liver by approx. 25%, but it had no effect on the computed liver content, because liver weight fell by 26% (lines 3 and 8, columns 2–5). The 0%-casein diet decreased the computed liver concentration and content of total BCDC by approx. 20% and decreased liver weight by 7%.

The results of measurement of citrate synthase specific activities in mitochondria and liver in Table 1 suggest that starvation and 0%-casein diet modify the liver concentration of total BCDC by effects on the liver concentration of mitochondria, whereas 24%-protein diet increases liver total BCDC by increasing mitochondrial specific activity. This suggestion has been confirmed by measurements of mitochondrial and liver specific activities of citrate synthase, GluDH, 2-ogDH and NAD-ICDH, the results of which are given in Table 2. Two of these enzymes (GluDH and citrate synthase) are mitochondrial matrix enzymes, and two (2-ogDH and NAD-ICDH) are mitochondrial membrane enzymes. Starvation increased liver specific activities of all four enzymes but had no effect on their mitochondrial specific activities. The 0%-casein diet increased mitochondrial specific activities of GluDH, 2-ogDH and NAD-ICDH, but not that of citrate synthase, and decreased liver specific activity of citrate synthase, but not those of GluDH, 2-ogDH or NAD-ICDH. Starvation increased mitochondrial concentration (by approx. 25%), and 0%-casein diet decreased it by approx. 20%. Liver activities of GluDH, 2-ogDH and NAD-ICDH were maintained on the protein-free diet, notwithstanding the decreased concentration of mitochondria, by a 25–50%

Table 2. Activities of L-glutamate dehydrogenase (GluDH), 2-oxoglutarate dehydrogenase complex (2-ogDH), NAD-isocitrate dehydrogenase (NAD-ICDH) and citrate synthase (CS) in rat liver and rat liver mitochondria

The details of preparation of extracts of liver and of liver mitochondria (three washes) and of spectrophotometric assays of enzyme activities were as in the Experimental section, except that ox serum was omitted from the extraction buffer. Livers from fed and starved rats (17%-protein diet) were compared on the same days as were livers from rats fed 24%- or 0%-protein diets. Liver mitochondrial concentrations (mg of mitochondrial protein/g of liver) were calculated from [liver enzyme activity (units/g)]/[mitochondrial enzyme activity (units/mg of protein)]. Results are means $\pm$ S.E.M.; the numbers of livers analysed are shown in parentheses. * $P < 0.01$, † $P < 0.02$, ‡ $P < 0.05$ for effect of starvation or 0%-casein diet. For other differences $P > 0.05$.

Enzyme (units)	Diet ...		17% protein		24% protein		0% protein	
	Rats ...		Fed	Starved (48 h)	Fed		Fed	
GluDH (units/g of liver)		196 $\pm$ 7.3 (6)		270 $\pm$ 13 (6)*	211 $\pm$ 13.1 (4)		214 $\pm$ 7.4 (4)	
GluDH (units/mg of mitochondrial protein)		3.4 $\pm$ 0.48 (6)		3.2 $\pm$ 0.18 (6)	3.7 $\pm$ 0.11 (4)		4.6 $\pm$ 0.26 (4)†	
2-ogDH (units/g of liver)		3.8 $\pm$ 0.04 (6)		4.4 $\pm$ 0.12 (4)*	3.8 $\pm$ 0.15 (4)		3.5 $\pm$ 0.37 (4)	
2-ogDH (munits/mg of mitochondrial protein)		64.8 $\pm$ 2.9 (4)		58.1 $\pm$ 1.5 (4)	62.4 $\pm$ 4.08 (4)		87.4 $\pm$ 5.35 (4)‡	
CS (units/g of liver)		14.3 $\pm$ 0.31 (6)		17.9 $\pm$ 0.32 (6)*	13.4 $\pm$ 0.41 (4)		9.6 $\pm$ 0.62 (4)*	
CS (munits/mg of mitochondrial protein)		230 $\pm$ 3.3 (6)		229 $\pm$ 2.8 (6)	230 $\pm$ 8.39 (4)		215 $\pm$ 10.8 (4)	
NAD-ICDH (munits/g of liver)		664 $\pm$ 19 (6)		881 $\pm$ 27 (6)*	819 $\pm$ 96 (4)		1023 $\pm$ 171 (4)	
NAD-ICDH (munits/mg of mitochondrial protein)		17.4 $\pm$ 1.8 (4)		15.3 $\pm$ 1.3 (4)	14.3 $\pm$ 2.1 (4)		21.5 $\pm$ 2.4 (4)‡	
Concn. (mg of mitochondrial protein/g of liver)								
GluDH		60.9 $\pm$ 2.5 (6)		84.4 $\pm$ 1.6 (6)*	56.3 $\pm$ 2.9 (4)		46.2 $\pm$ 2.3 (4)‡	
2-ogDH		59.6 $\pm$ 1.8 (4)		75.0 $\pm$ 3.2 (4)†	61.5 $\pm$ 2.5 (4)		39.9 $\pm$ 1.9 (4)*	
CS		62.2 $\pm$ 1.2 (6)		77.8 $\pm$ 1.3 (6)*	58.3 $\pm$ 2.8 (4)		44.4 $\pm$ 1.5 (4)*	
NAD-ICDH		39.0 $\pm$ 3.1 (4)		62.4 $\pm$ 2.5 (4)*	57.2 $\pm$ 1.9 (4)		47.6 $\pm$ 2.3 (4)†	

increase in the mitochondrial specific activities of these enzymes. The estimates of liver mitochondrial concentration with citrate synthase, GluDH and 2-ogDH agreed closely; values with NAD-ICDH tended to be lower (Table 2).

Zhang *et al.* [9] found no significant effect of starvation on rat liver mitochondrial concentrations of citrate synthase or total BCDC. They found also that starvation increased specific activity of whole liver total BCDC by 40%, but failed to detect any increase in whole-liver specific activity of citrate synthase. The possible basis for such a dissociation between whole liver and mitochondria, in respect of the specific activities of BCDC and citrate synthase, is not clear. Zhang *et al.* [9] did not give liver weights or comment on the possible effect of starvation on liver mitochondrial concentration. In rats fed on 0%-casein diet, factors which might contribute to the maintenance of liver weight, and thereby to a lower liver concentration of mitochondria, include deposition of fat and glycogen [22].

The specific activities of total BCDC in rat liver that we have obtained by the mitochondrial method (Table 1, line 2) are approx. 25% higher than those obtained by radioactive assay by Harris *et al.* [7] and by Zhang *et al.* [9]. Our own estimates and those in refs. [7,9] are approximately twice those obtained with a radioactive assay by Solomon *et al.* [8]. The effects of dietary variation in all of these studies are comparable.

The percentage of BCDC in the active form is shown in line 4 of Table 1. Our own estimates show no significant differences from those in refs. [7–9]. In earlier

studies from our laboratory [5,6], starvation was found to decrease the percentage of active BCDC. This has not been confirmed by the present results, which agree with those of Harris *et al.* [7]. The reason for this discrepancy has not been resolved. The original observations, which were confirmed in our laboratory by more than one investigator, gave results which in all respects were similar to those of low-protein diet. Whether the starved rats in the earlier studies had access to non-protein feed cannot be ascertained, but an effect of sawdust or wood shavings, which might have been used as bedding, has been excluded (results not shown).

General discussion and conclusions

The present study has identified a number of parameters that must be measured in order to define the contribution of the liver to whole-body active BCDC and the mechanisms regulating the liver content of the active form of BCDC. These comprise the mitochondrial specific activity of total BCDC, the liver concentration of mitochondria, liver weight, the percentage of BCDC in the active form and the liver concentration of BCDC. We have preferred to measure BCDC (total and active form) in extracts of mitochondria and to compute whole-liver concentrations of BCDC and of mitochondria from mitochondrial and liver concentrations of citrate synthase. We prefer this method, because the rate of BCDC holocomplex reaction can be monitored by spectrophotometric assay of NADH. We have confirmed the validity of the use of citrate synthase for measurement of the liver concentration of mitochondria by comparison

with the three other mitochondrial marker enzymes. We have also given evidence that the mitochondrial method for measurement of percentage of active BCDC *in vivo* is valid, notwithstanding the criticisms by Zhang *et al.* [9].

The mitochondrial method has been improved by a number of important modifications. The relationship between BCDC and citrate synthase activities has been more accurately defined; use of dichloroacetate in place of ketoleucine has improved the accuracy of BCDC assays by allowing more accurate determination of blank rates; and the estimation of liver citrate synthase has been improved by use of fresh liver and a more effective battery of proteinase inhibitors.

The present study has shown that increasing the protein content of diet from 17 to 24% increases the liver content of total and active BCDC by increasing the mitochondrial specific activity of total BCDC. This might suggest that the mechanism of the effect of 24%-protein diet involves increased synthesis of the component enzymes of the complex, and/or decreased degradation or perhaps an increased rate of mitochondrial uptake of these components or their precursors. This is perhaps the first unequivocal demonstration that dietary protein can modify the mitochondrial concentration and liver content of total BCDC activity. The present study has also shown that starvation increases liver total BCDC concentration by increasing liver mitochondrial concentration, but not liver content. The mechanism involves a decrease in liver weight, presumably as a result of the loss of glycogen, other proteins and water. The 0%-protein diet decreased liver total BCDC concentration and content by decreasing liver concentration and content of mitochondria. These observations, and the further finding that starvation has no effect on the percentage of active BCDC in liver, can account for major discrepancies between results published in refs. [5,6] and [7,8].

The adventitious observation that the liver activities of GluDH, 2-ogDH and NAD-ICDH were maintained on protein-free diet by an increase in their mitochondrial specific activities is of possible biological significance, because of the central role of glutamate and 2-oxo-glutarate in many aspects of amino acid metabolism.

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