# Developmental pattern of branched-chain 2-oxo acid dehydrogenase complex in rat liver and heart

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The developmental pattern of the branched-chain 2-oxo acid dehydrogenase complex was examined in the liver and heart of the rat throughout the suckling period. Basal activity and total activity of the complex were measured as a function of age. The hepatic enzyme activity increased dramatically and was 100% active (dephosphorylated) during the suckling period. The level of protein kinase associated with the complex was particularly low at birth, but like the complex increased throughout the suckling period. The level of heart enzyme also increased as a function of age, but only about 30–45% of the enzyme was active throughout the suckling period. Very low protein levels of liver and heart branched-chain 2-oxo acid dehydrogenase were detected by immunoblot analysis in newborn rats. The mRNA levels for the liver E1$\alpha$, E1$\beta$, and E2 subunits in newborn rat were 30%, 19%, and 4% of adult levels respectively. The capacity of the neonatal rat for oxidizing leucine *in vivo* was low at birth and increased with age. 4-Methyl-2-oxopentanoate was more toxic when given to newborn and 3-day-old pups than 21-day-old pups, as expected from the relative capacities of their tissues to dispose of branched-chain 2-oxo acids by oxidation. Force-feeding suckling rats a protein-free artificial milk formula resulted in partial inactivation of the hepatic branched-chain 2-oxo acid dehydrogenase complex, indicating that the liver of the suckling rat can adapt to conserve branched-chain amino acid residues during periods of protein deficiency.

## INTRODUCTION

Branched-chain amino acids (BCAA) are essential for protein synthesis and can also serve as oxidizable substrates in most tissues of the body. The branched-chain 2-oxo acid dehydrogenase complex (BCODC), an intramitochondrial enzyme, catalyses the committed step in the pathway of degradation of BCAA. Regulation of BCODC activity by phosphorylation/dephosphorylation has been established as important in animals starved of dietary protein [1–3]. Inactivation of hepatic BCODC of protein-starved animals serves to conserve BCAA for protein synthesis. Inhibition by branched-chain 2-oxo acids of the kinase responsible for phosphorylation and inactivation of BCODC is the only established mechanism for the regulation of the activity state of the complex [4,5]. A major purpose of the present work was the determination of whether regulation of BCODC by reversible phosphorylation is operational in the newborn rat.

Although essential for protein synthesis, it is also apparent that excessive amounts of BCAA are toxic to animals. Maple-syrup-urine disease (MSUD) is caused by a defect in the catabolism of BCAA at the level of BCODC [6]. The severity of this disease demonstrates the importance of the BCAA catabolic pathway for the disposal of these amino acid residues. Polled Hereford cattle from Australia provide the only documented animal model for MSUD [7]. Newborn human infants, as well as newborn calves with classic MSUD, appear normal at birth but rapidly develop a severe neurological disorder, indicating that a functional BCODC must be either present at birth or induced within a few days of birth in humans and cattle in order to avoid the consequence of failure to dispose of excessive amounts of BCAA [6,8]. In this regard, it is surprising that previous developmental studies of BCODC in the rat have indicated very low activity at birth and a relatively slow increase in activity during the suckling period [9–11]. Since the latter work was carried out prior to full appreciation of the importance of regulation of BCODC by reversible phosphorylation and full development of assay systems for total activity as opposed to flux measurements, we have re-examined the developmental pattern of BCODC.

## MATERIALS AND METHODS

### Materials

[1-$^{14}$C]Leucine, $^{125}$I-protein A, and radioactive nucleotides were obtained from New England Nuclear Corp., Boston, MA, U.S.A. A random-primed DNA labelling kit for labelling cDNA probes was from United States Biochemical Corp., Cleveland, OH, U.S.A. RNAzol was from Cinna/Biotex Inc., Houston, TX, U.S.A. All chemicals and bovine heart dihydrolipoamide reductase were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Pregnant Wistar rats (Harlan Industries, Indianapolis, IN, U.S.A.) were housed individually in maternity cages in a temperature- and light-controlled room. Rats were fed a Purina Rodent Laboratory Chow 5001 diet *ad libitum*. Pups were removed for the *in vivo* study or for tissue collection at 0, 7, 14, and 21 days of age.

### Assay of BCODC and BCODC kinase activity

The basic procedure used for the assay of BCODC is based on that described by Goodwin et al. [12]. Tissues were quickly removed, freeze-clamped at the temperature of liquid nitrogen and extracted for enzyme assay. The enzyme was extracted in a buffer described previously [12] and concentrated by precipitation with 9% (w/v) polyethylene glycol (PEG) and pre-incubated with a broad-specificity phosphoprotein phosphatase [5] for complete activation of the complex by dephosphorylation. Assay of BCODC without phosphatase treatment allowed the deter-

Abbreviations used: BCAA, branched-chain amino acids; BCODC, branched-chain 2-oxo acid dehydrogenase complex; MSUD, maple-syrup-urine disease; PEG, polyethylene glycol.

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mination of enzyme activity as it existed in the tissue, whereas phosphatase treatment provided a measurement of total enzyme activity. Enzyme activity is expressed in terms of $\mu$mol of NADH/min per g wet wt. Three different litters and two pups from each litter were analysed.

The activity of BCODC kinase is expressed as the first-order rate constant of inactivation of the dehydrogenase, determined from the slope of a semi-logarithmic plot of remaining activity as a function of incubation time [13].

### Western-blot analysis and immunoquantitative assay of BCODC E2-subunit protein

The PEG-concentrated BCODC was resuspended in an SDS solution [4% (w/v) SDS/0.125 M Tris/HCl, pH 6.8/20% (w/v) glycerol/1.5 mg/ml dithiothreitol] to correspond to 40 mg of original wet wt. of tissue/ml [12]. The procedure used for the Western-blot analysis was as described in [7]. Briefly, samples were subjected to SDS/PAGE and transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with a mixture of polyclonal antisera raised against the E1 and E2 components of BCODC complex. Immunoreactive proteins were detected with the AuroProbe BLplus kit (Janssen Life Science Products, Olen, Belgium) following the manufacturer's instructions. For the immunoquantitative assay, samples were applied to a dot-blot apparatus in serial dilutions. A monospecific polyclonal antibody raised against the BCODC E2 subunit was used and followed by $^{125}$I-labelled protein A detection. Quantification was done by densitometry (Helena Laboratories, Beaumont, TX, U.S.A.).

### Northern-blot analysis of the BCODC mRNA levels

Total cellular RNA was extracted from freeze-clamped tissue by the RNAzol method (Cinna/Biotex Laboratories International Inc., Houston, TX, U.S.A.) following the instructions of the manufacturer. Northern blotting was conducted by a standard protocol [14]. Rat BCODC E1$\alpha$, E1$\beta$, E2, and actin cDNAs were used as probes for the hybridization. Radioactivity associated with the bands was determined quantitatively with an AMBIS $\beta$-scanner.

### Force-feeding experiments with suckling rats

Pups of 0, 3, and 21 days of age were force-fed with sodium 4-methyl-2-oxopentanoate (30 $\mu$mol/g of body wt. per feeding); the same dose of sodium acetate was given to the control animals. Pups were returned to their mother and observed for 6 h. In another experiment, newborn rats were force-fed a dose of 4-methyl-2-oxopentanoate (15 $\mu$mol/g of body wt. per feeding) twice a day at an interval of 12 h for 7 days. Livers were rapidly removed, freeze-clamped and extracted for use in enzyme assays. In yet another experiment, a protein-free formula was administered to 7-day-old pups (0.25 ml/h) [15] for 14 h. These pups remained isolated from their mothers. Control animals were force-fed a complete artificial formula, consisting of 15% (w/v) non-fat dry milk, 10% (w/v) fat (soybean oil), 7% (w/v) lactose, 0.209% (w/v) $MgCl_2$, 0.244% (w/v) $CaCl_2$, 0.588% (w/v) $Ca(CH_3COO)_2$, 0.523% (w/v) $KH_2PO_4$, 0.386% (w/v) $NaH_2PO4$, and 0.007% (w/v) $ZnSO_4$ [16,17]. The protein-free formula was prepared without the non-fat dry milk.

### *In vivo* oxidative capacity of the suckling rat for leucine

The procedure was based on that described by Ward et al. [18]. [1-$^{14}$C]Leucine (1 $\mu$Ci, 5 mCi/mmole) was injected intraperitoneally into each pup. Two pups were then placed in a 500 ml flask clamped in a 32 °C water bath. Air was forced through the flask at a rate of 500 cm$^3$/min. $^{14}CO_2$ of the air passing through the flask was trapped in an alkaline buffer [mixture of ethanolamine and methylcellosolve (1:2, v/v)]. Two traps of 50 ml each, connected in series, were used. Aliquots (1 ml) were removed from the traps for counting every 20 min, for 4 h. At each sampling time, 1 ml of the alkaline solution was added back to the traps. The percentage of released radioactivity was plotted as a function of time. Results were collected for two pups of each litter and at least three separate litters for the same age rats were studied. Results are expressed as means ± S.E.M. with the $n$ value corresponding to the number of pairs of animals used at each age.

## RESULTS

The activity of BCODC was extremely low in the liver of the newborn rat and could not be detected in the heart (Figure 1). Treatment of tissue extracts with a broad-specificity phosphoprotein phosphatase, under conditions established to activate BCODC by dephosphorylation [5], had no effect on liver enzyme activity. This finding indicates that the very low amount of BCODC present in rat liver at birth is all dephosphorylated and active. Liver BCODC activity increased throughout the suckling period (Figure 1), reaching adult levels at the age of weaning (21 days). Phosphatase treatment had no effect on the activity of liver BCODC throughout the suckling period (Figure 1). Thus, BCODC of the liver is completely dephosphorylated and active in the suckling rat, as demonstrated previously for mature rats on a chow diet (> 23% protein) [12]. Heart BCODC was barely detectable at birth and also increased in total activity as a function of age in the suckling rat (Figure 1), but in contrast with the liver, the increase with age was modest, reaching 34% of the adult total activity at the age of weaning. The complex was 30–40% active (dephosphorylated) throughout the suckling

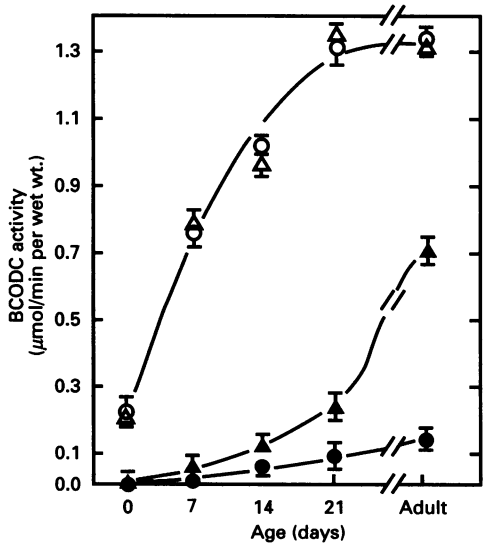


**Figure 1 Activity and activity state of liver and heart BCODC in the developing rat**

Two pups from each litter and three different litters for each age group were used for collecting the results. The results are expressed as means ± S.E.M. Basal enzyme activity was measured before phosphatase treatment; total enzyme activity after phosphatase treatment: ○, hepatic enzyme basal activity; △, hepatic enzyme total activity; ●, heart enzyme basal activity; ▲, heart enzyme total activity.

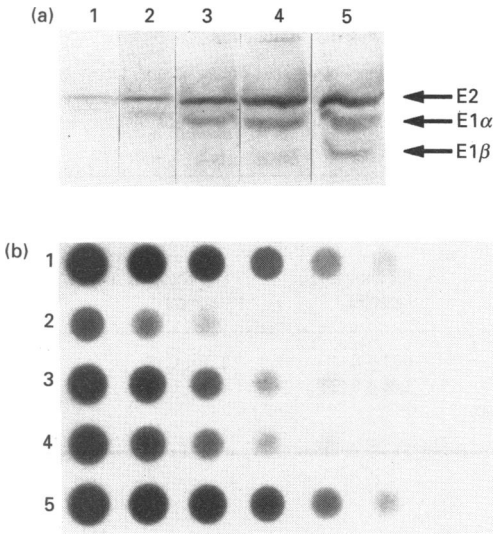


**Figure 2 Western-blot analysis of hepatic BCODC complex and quantification of the protein mass of the E2 subunit of BCODC in the liver and heart of the rat**

(**a**) Tissue extracts were prepared as described in the Materials and methods section. Same amount of sample extract (corresponding to 0.3 mg wet wt.) was loaded for SDS/PAGE followed by immunoblot analysis with E1 and E2 antibodies. Lanes 1–5 correspond to liver homogenates of 0-day-old, 7-day-old, 14-day-old, 21-day-old, and an adult rat respectively. (**b**) Tissues extracts of newborn and adult rats (175–200 g body wt.), as indicated, were spotted on nitrocellulose paper at 2-fold serial dilutions corresponding to the same wet wt. The filters were immunoblotted with polyclonal antibodies against the E2 subunit of BCODC and detected by $^{125}$I-labelled protein A. Lane 1, standard BCODC.; lane 2, tissue extract from newborn rat heart; lane 3, tissue extract from adult rat heart; lane 4, tissue extract from newborn rat liver; lane 5, tissue extract from adult rat liver.

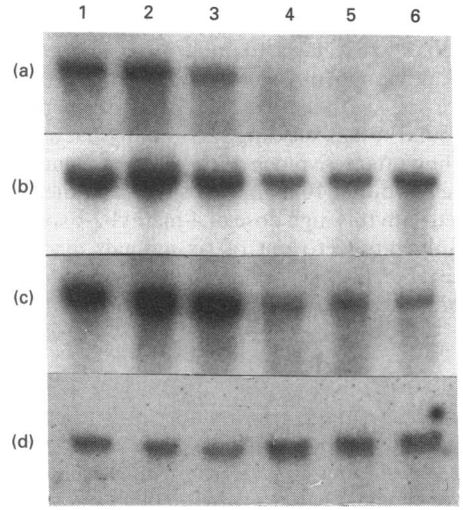


**Figure 3 Northern-blot analysis of the content of mRNAs for E2, E1α, and Eβ subunits in liver of the newborn and adult rats**

Total RNA samples were extracted from newborn and adult rat liver and electrophoresed and blotted on to nitrocellulose paper. The membranes were probed with labelled cDNAs for rat BCODC E2, E1α, E1β, and actin. Lanes 1–3 contained liver total RNA from three different adult rats; lanes 4–6 contained liver total RNA from newborn rats of three different litters. Rat BCODC E2, E1α, E1β, and actin cDNAs were used as probes in (**a**), (**b**), (**c**), and (**d**) respectively.

period, a value greater than that typically found in the heart of adult animals (5–18 % in the active form) [12,19] (Figure 1).

An age-dependent increase of the hepatic BCODC complex

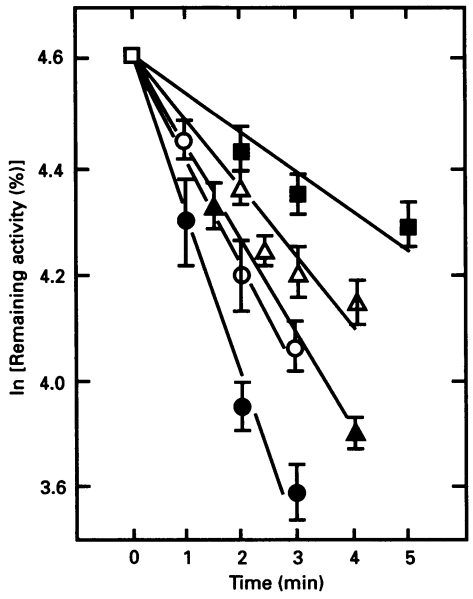


**Figure 4 Change in BCODC kinase activity as a function of age**

Plotted on the ordinate is the ln of the percentage of BCODC activity remaining as a function of time after initiation of kinase activity with the addition of ATP. The slopes of the curves correspond to the activity of the BCODC kinase. Data points of each line correspond to the mean ± S.E.M. for three animals: ■, newborn; △, 7 days old; ▲, 14 days old; ○, 21 days old; ●, adult.

was also observed by Western-blot analysis (Figure 2a). The quantity of BCODC in the liver and heart, detected by immunoblot analysis, was found to be proportional to the total activity of the enzyme measured in the heart and liver of newborn and adult rats. Thus, almost undetectable levels of E2-subunit protein for the complex was found in the heart of the newborn relative to the adult (Figure 2b), and only about 25 % as much E2 protein was found in the liver of the new born relative to the adult (Figure 2b).

Northern-blot analysis was used to quantify mRNA levels of BCODC subunits in liver of newborn and adult animals (Figure 3). The quantities of RNA subjected to electrophoresis were comparable, as indicated by ethidium bromide staining of ribosomal RNA bands (results not shown). The mRNA level for the E2 subunit was barely detectable in newborn rats (4 ± 2 % of the adult liver level, as measured by β-scanner analysis). E1α and E1β subunit mRNA levels were also lower, measuring 30 ± 5 % and 19 ± 3 % of the respective adult levels. Actin mRNA levels were higher in the newborn liver than the adult, confirming that the findings are not due to differences in quantities of RNA subjected to electrophoresis.

Since BCODC of the liver was found to be completely active throughout the suckling period, it was of interest to determine whether BCODC kinase activity was expressed in the liver of the suckling rat. The only assay currently available for BCODC kinase activity measures the activity of the enzyme tightly associated with the complex [13]. Liver BCODC kinase activity was low but present in the newborn rat, and increased throughout the suckling period to a level of about 60 % of the adult level at the age of weaning (Figure 4). First-order rate constants for kinase activity were 0.08 $min^{-1}$ for newborn rats, 0.13 $min^{-1}$ at 7 days, 0.18 $min^{-1}$ at both 14 and 21 days, and 0.30 $min^{-1}$ for adult rats.

The technique of a single injection of [1-$^{14}$C]leucine [18,20,21] was used to assess the relative capacity of the suckling rat to

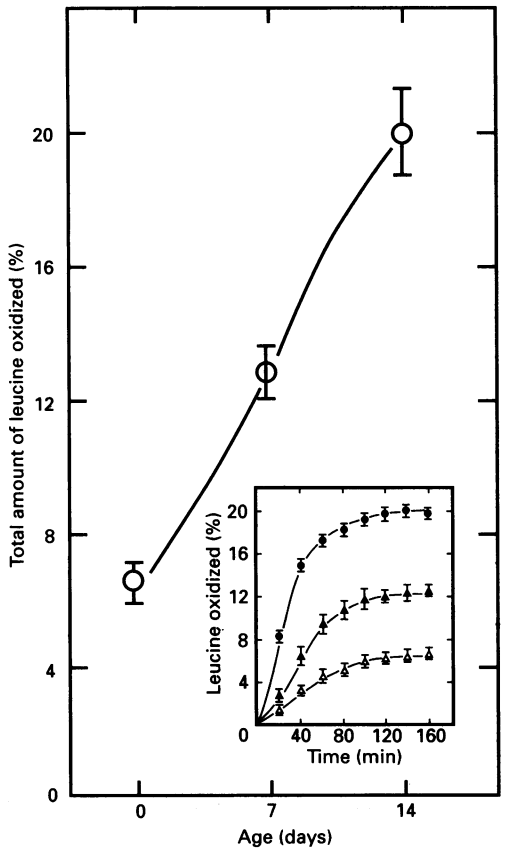


**Figure 5 The capacity *in vivo* of the neonatal rat for oxidizing leucine**

[1-$^{14}$C]Leucine was administrated and $^{14}CO_2$ collected as described in the Materials and methods section. The insert shows the percentage of total radioactivity injected that was released as $^{14}CO_2$ plotted as a function of time: △, newborn; ▲, 7 days old; ●, 14 days old. The cumulative percentage of dose administrated, determined from the results of the insert, is plotted as a function of age. Results were collected from rats from three different litters. Results are expressed as mean ± S.E.M.

**Table 1 Effect of protein-free formula on liver BCODC activity and activity state of 7-day-old rats**

The artificial milk formulae were administered hourly for 14 h. All values are given as means ± S.E.M. for six animals. *$P < 0.01$ versus control by unpaired Student's *t*-test.

| Milk formula | Liver BCODC activity (μmol/min per g wet wt.) Pre-phosphatase treatment | Post-phosphatase treatment | BCODC activity state (%) |
|---|---|---|---|
| Complete | 0.73 ± 0.15 | 0.69 ± 0.11 | 105 ± 6 |
| Protein-free | 0.48 ± 0.09* | 0.71 ± 0.20 | 67 ± 6* |

oxidize branched-chain amino-acid residues versus the ability to utilize these compounds for protein synthesis. As demonstrated previously for mature rats [18], radioactive $CO_2$ production from [1-$^{14}$C]leucine was rapid for the first 80 min after administration but practically ceased by 3 h (Figure 5, insert). In the latter time period, the newborn rat converted only 2% of administered [1-$^{14}$C]leucine into $^{14}CO_2$ (Figure 5), presumably incorporating

**Table 2 Effect of chronic 4-methyl-2-oxopentanoate administration on liver BCODC activity and activity state**

Newborn rats were given 15 μmol of 4-methyl-2-oxopentanoate/g body wt twice daily at 12-h intervals for 7 days. Control animals were given same dose of sodium acetate. All values are given as means ± S.E.M. for six animals. *$P < 0.01$ versus control by unpaired Student's *t*-test.

| Treatment | Liver BCODC activity (μmol/min per g wet wt.) Pre-phosphatase treatment | Post-phosphatase treatment | BCODC activity state (%) |
|---|---|---|---|
| Control | 0.71 ± 0.11 | 0.70 ± 0.13 | 102 ± 6 |
| 4-Methyl-2-oxopentanoate | 1.05 ± 0.13* | 1.05 ± 0.22* | 100 ± 8 |

the balance of the radioactive leucine into protein. The percentage of the leucine residues oxidized increased sharply as a function of age (Figure 5), in agreement with the age-dependent increase in expressed BCODC activity of tissues demonstrated above.

Liver BCODC basal activity was decreased significantly in suckling rats force-fed a protein-free formula every hour for 14 h (Table 1). Phosphatase treatment activated the complex to the same level of activity as that of control suckling rats (Table 1), thereby establishing that the low activity of BCODC in protein-starved suckling rats is a consequence of inactivation of the complex by phosphorylation.

Chronic force-feeding of suckling rats with 4-methyl-2-oxopentanoate resulted in an increase in liver BCODC activity (Table 2). Since phosphatase treatment had no effect on enzyme activity in either the control or treated animals this finding cannot be explained by the activity state of BCODC and, therefore, probably reflects an adaptive increase in enzyme mass.

Force-feeding 30 μmol of 4-methyl-2-oxopentanoate/g of body wt., i.e. twice the quantity used in the experiment described above, was found to kill suckling rats rapidly. Newborn and 3-day-old suckling rats were much more sensitive (nine out of nine animals and eight out of 10 animals respectively, died within 6 h when force-fed with this high dose of 4-methyl-2-oxopentanoate) than 21-day-old rats (zero out of six animals died), probably reflecting the relative capacities of these animals for oxidative disposal of branched-chain 2-oxo acids.

## DISCUSSION

The developmental patterns of a number of liver enzymes have been characterized in the suckling rat [22–24]. BCODC is a relatively late-developing enzyme, showing a somewhat more dramatic developmental pattern between birth and weaning than the pyruvate dehydrogenase complex [25], the urea-cycle enzymes [26,27], and the increase in mitochondrial content of liver [28,29]. The function of BCODC is to catalyse the first irreversible step in the disposal of BCAA, all of which are essential for protein synthesis, but yet toxic if allowed to accumulate. The low expressed activity in the newborn rat indicates little need exists at birth for the disposal of these amino-acid residues. Practically all of the BCAA available in milk consumed by the newborn rat must be utilized for protein synthesis. The relatively low conversion rate of [1-$^{14}$C]leucine into $^{14}CO_2$ by newborn rats supports this interpretation. Rat milk must provide the suckling rat with BCAA in the right ratios relative to other amino-acid residues, as well as total calories needed for optimum body growth. This

developmental pattern for BCODC of the rat is different from that of the sheep, bovine and man. Considerable BCODC activity has been shown to be present in the liver of the sheep fetus before birth, and the oxidation of BCAA has been studied in some detail in the sheep fetus [30]. Less appears to be known about the developmental pattern of BCODC in the bovine and human, but the blood leucine concentration of bovine calves and human infants with MSUD can be abnormally high within hours of birth [6,8]. Also, the studies *in vivo* on the leucine metabolism in human newborns [31] showed that leucine oxidation in newborn infants was at least at the same level as in adults, suggesting an important role for BCODC in disposal of excess BCAA in tissues of normal calves and human infants.

An age-dependent increase in BCODC activity during neonatal development of the rat has been documented previously by others [9–11]. The work of May et al. [9] first described the developmental pattern for the liver, but whether the increase throughout the suckling period was due to a change in enzyme mass or covalent modification was not investigated. Veerkamp et al. [10] and Grogan et al. [11] measured enzyme activity by a flux method [32] and concluded that the hepatic enzyme was largely in its active, dephosphorylated form throughout postnatal development. The present work further documents the increase in hepatic enzyme activity throughout the suckling period of development and confirms that the increase in enzyme activity in the liver is due to an increase in expressed amount of BCODC subunits rather than a change in activity (phosphorylation) state of the enzyme. The low, but not strictly proportionately low, mRNA levels for the BCODC subunits may explain why the amount of BCODC protein is low in the newborn and suckling rats. Whether the transcription rate of the genes involved or mRNA stability is responsible for the low message levels remains to be investigated. BCODC kinase activity was low in the liver at birth and increased only slowly throughout the suckling period, and this may explain why the enzyme was always found in its completely activated state. That functional kinase was present in the liver of newborn and suckling rats was demonstrated by direct enzyme assay and with the finding of a partial inactivation of the hepatic complex upon feeding suckling rats a protein-free formula for a few hours. Whether the increase in kinase activity reflects a change in enzyme mass, intrinsic enzyme activity, or binding to the complex is unknown.

Like liver BCODC, the activity of the heart enzyme was low at birth, but in contrast with the liver, much of the heart enzyme was in its phosphorylated inactive form throughout the suckling period. Similar results on heart actual and total activities were described by Veerkamp et al. [10]. Grogan et al. [11] reported similar results for the skeletal muscle and brain for 10-day-old to 30-day-old suckling rats. The proportion of the heart enzyme that was dephosphorylated and active was greater during the suckling period than observed with the heart enzyme of mature animals [12,19].

Force-feeding of 4-methyl-2-oxopentanoate to newborn rats increased BCODC activity, presumably because of an increase in amount of enzyme, although the actual mechanism involved was only shown not to involve a change in activity (phosphorylation) state of BCODC. These findings stand in contrast with those of Grogan et al. [11] who did not find an increase in BCODC activity in response to daily leucine dosing. The use, in the present study, of 4-methyl-2-oxopentanoate (rather than leucine) at a higher daily dose (30 $\mu$mol/g body wt. versus 10 $\mu$mol/g body wt.) may explain the difference in findings. Force-feeding a larger amount of 4-methyl-2-oxopentanoate in one dose proved toxic to very young suckling rats, presumably because of their limited capacity to oxidize branched-chain oxo acids. This finding suggests that an animal model for MSUD could perhaps be developed by force-feeding branched-chain oxo acids to suckling rats in amounts exceeding their enzymic capacity for oxidation of these compounds.

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