Developmental changes in rat liver branched-chain 2-oxo acid dehydrogenase

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Branched-chain 2-oxo acid dehydrogenase catalyses the first irreversible step in the degradation of the branched-chain amino acids leucine, isoleucine and valine. With specifically labelled 4-methyl-2-oxo[1-¹⁴C]pentanoate as substrate, the enzyme's activity was measured in rat liver homogenates. Activity (per g wet wt. of liver or per mg of protein) increased most rapidly during the perinatal period (2 days before to 1 day after birth), reaching approximately adult values by the time of weaning. The apparent $V_{\max}$ of the enzyme increased with age, but its K_m appeared unchanged. The data suggest that hepatic branched-chain 2-oxo acid dehydrogenase is induced or activated during the perinatal period. The enzyme's activity at birth was unaffected by maternal diabetes, or by treating the mother with pharmacological doses of corticosterone or 3,3',5-tri-iodothyronine, during the last 5 days of pregnancy.

The oxidative decarboxylation of the branched-chain amino acids is the first committed step in their degradation. Congenital absence of the mitochondrial enzyme catalysing this reaction, the branched-chain oxo acid dehydrogenase (EC 1.2.4.4), results in a severe metabolic derangement which is characterized by accumulation of the branched-chain amino acids and oxo acids in tissues and by neurological abnormalities. Patients affected by this disorder appear normal at birth, but then manifest symptoms and signs some 5–7 days later (Dancis & Levitz, 1972). The delayed phenotypic appearance of the disease suggests that branched-chain 2-oxo acid dehydrogenase may not be fully active during foetal life and during the immediate perinatal period. The tissue concentrations of other oxidative mitochondrial enzymes, such as pyruvate dehydrogenase (Bailey *et al.*, 1976), cytochrome oxidase (Lang, 1965), succinate dehydrogenase (Potter *et al.*, 1945) and β -hydroxybutyrate dehydrogenase (Hommes *et al.*, 1971), have been shown to be initially low at birth and to increase during the first 2 weeks of life.

The present paper describes the perinatal development of hepatic branched-chain 2-oxo acid dehydrogenase in normal rat pups and in pups exposed to an abnormal hormonal milieu *in utero*. Parts of

this paper were presented at the 1980 Meeting of the Federation of American Societies for Experimental Biology (May & Buse, 1980).

Materials and methods

Sources of materials

L-[1-¹⁴C]Leucine was obtained from New England Nuclear Corp., Boston, MA, U.S.A., and 4-methyl-2-oxo[1-¹⁴C]pentanoate was prepared therefrom by the amino acid oxidase method of Rudiger *et al.* (1972). The radiolabelled oxo acid was stored in 25 μ l batches (4 μ Ci/ml in 0.01 M-HCl) at -70°C , and showed no evidence of decomposition on t.l.c. (Magasanik & Umbarger, 1950) up to six months of storage. NAD⁺, CoA (lithium salt), thiamin pyrophosphate, sodium 4-methyl-2-oxopentanoate, Tween 20, cytochrome *c*, leupeptin and T₃ were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Nonidet P-40 was supplied by Gallard-Schlesinger Chemical Manufacturing Corp., Carl Place, NY, U.S.A., and NADH:lipoamide oxidoreductase (EC 1.6.4.3) was from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Corticosterone was a product of Steraloids, Pawling, NY, U.S.A. Streptozotocin was kindly supplied by Dr. William Dulin, Upjohn Research Laboratories, Kalamazoo, MI, U.S.A.

Animal care

Pregnant rats of the Sprague-Dawley strain with known date of conception were obtained from

Abbreviation used: T₃, 3,3',5-tri-iodothyronine.

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Charles River Breeding Laboratories, Newfield, NJ, U.S.A. Rats had access to food and water *ad lib*. Diabetes was induced on day 12 of pregnancy by injecting streptozotocin (60 mg/kg) into the tail vein of rats after starvation for 18 h. Control and experimental animals were weight-matched at the time of injection. Animals were classified as diabetic if they had 2+ to 3+ (Tes-Tape) glucosuria and plasma glucose concentrations exceeding 300 mg/dl. Corticosterone (1 mg/day) suspended in peanut oil was injected subcutaneously from days 17 to 21 of pregnancy. T_3 was dissolved in 0.01 M-NaOH, further diluted in 0.9% NaCl, and injected subcutaneously (150 μ g/kg body wt. per day) from days 17 to 21 of pregnancy. Controls for both treatment groups were injected with the appropriate diluent. Pregnant rats and their offspring were decapitated by guillotine. Diabetic and hormone-treated dams were killed on day 22 of pregnancy. Pups were immediately externalized by abdominal uterotomy and kept in a humidified, aerated chamber at 32°C till killed. Mixed arterio-venous blood was collected into heparinized tubes from severed neck vessels and the plasma was used for glucose and thyroid-hormone measurements.

Tissue preparation and assay

Immediately after the animals were killed, portions of liver were removed, weighed, minced and homogenized with a Teflon pestle in a Potter-Elvehjem homogenizer in an iso-osmotic buffer (7 ml/g of tissue) containing 220 mM-mannitol, 70 mM-sucrose, 0.1 mM-EGTA, 2 mM-Mops (4-morpholinepropanesulphonate) and fatty acid-free bovine serum albumin (0.5 mg/ml), neutralized to pH 7 at 4°C with NaOH. The homogenates were maintained at 4°C until initiation of the assays, within 30 min of death of the rats. In preliminary experiments the activity of the homogenates at 4°C was stable for at least 30 min.

In the standard assay, to 500 μ l of assay medium in a plastic tube at 37°C was added 50 μ l of homogenate, followed after 2 min by addition of 50 μ l of deaerated 2.4 mM-4-methyl-2-oxo[1- 14 C]-pentanoate (approx. 0.02–0.05 μ Ci) dissolved in assay buffer (final substrate concn. 0.2 mM). The tubes were sealed with a serum cap and incubated for 5 min at 37°C with shaking. Then 0.3 ml of Hyamine hydroxide was injected into a plastic cup suspended above the liquid, followed immediately by injection of 0.3 ml of 25% (w/v) trichloroacetic acid into the reaction mixture. Incubation and shaking were continued at 37°C for 60 min (Buse *et al.*, 1976), followed by transfer of the plastic cups to 5.8 ml of 0.5% (w/v) diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene for liquid-scintillation counting in a Beckman LS 7000 scintillation spectrophotometer, with a 75%

efficiency for 14 C. Counts were corrected for quenching by automatic external standardization or by the channels-ratio method and were expressed as d.p.m. All samples were counted for radioactivity for 10 min or until enough counts were accumulated to decrease the counting error to less than 2%. Each assay was done in triplicate; values were corrected for 14 CO₂ evolution in control tubes containing 50 μ l of assay buffer in place of homogenate. We have found that identical blank values were obtained with either heat-treated tissue (3 min at 90°C) or buffer; the latter was generally used for convenience. Blanks were less than 100 d.p.m.

The assay buffer contained (in mM): mannitol, 220; sucrose, 70; MgCl₂, 2; and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 2; pH 7.4 at 37°C. This buffer has been reported to maintain the morphological and functional integrity of liver mitochondria (Schnaitman & Greenawalt, 1968; May *et al.*, 1980). Enzyme activities at various substrate concentrations were measured in the standard assay buffer and in buffer supplemented with 0.1% (w/v) Nonidet P-40, 2 mM-NAD⁺, 0.5 mM-thiamin pyrophosphate and 0.8 mM-CoA.

A representative assay mixture in standard assay buffer containing 6 mg (50 μ l) of foetal liver homogenate (21 days gestation) released approx. 1000 d.p.m. of 14 CO₂ in 5 min. The s.d. for triplicate measurements was less than 10%. When livers from postnatal rats or 22-day foetuses were used, the decarboxylating activity was approximately twice as much in the detergent-containing assay buffer as in the standard assay buffer. However, liver homogenates from younger foetuses lost their decarboxylating activity when exposed to the detergent-containing buffer, and this was not prevented by supplementing the buffer with leupeptin (10 μ g/ml) or lipoamide dehydrogenase (1.7 munits/ml).

In both assay buffers 14 CO₂ release was proportional to protein concentration when homogenate addition was varied between 25 and 75 μ l (results not shown) and was linear with time between 1 and 7 min incubation (Fig. 1). Since in the detergent-containing buffer proteolysis or desaturation of endogenous lipoamide dehydrogenase (Pettit *et al.*, 1978) may occur, liver homogenates from 22-day foetuses were assayed in the detergent-containing buffer, supplemented with cofactors (see above) in the presence or absence of leupeptin (10 μ g/ml) or lipoamide dehydrogenase (1.7 munits/ml). Activities were measured as described in the standard protocol and were (in nmol/min per mg of protein): 2.37 ± 0.44 for controls, 2.40 ± 0.46 with leupeptin and 2.37 ± 0.42 with lipoamide dehydrogenase ($n = 4/\text{group}$). In a previous study, with freeze-thawed liver mitochondria from adult rats, the addition of leupeptin also failed to affect branched-

Fig. 1. Linearity of $^{14}\text{CO}_2$ production by foetal rat liver homogenates with incubation time

Homogenates were prepared from foetal rat livers of 20 (●), 21 (▲) and 22 days (■) gestation; 6 mg of tissue was incubated at 37°C with 0.2 mM 4-methyl-2-oxo[1- ^{14}C]pentanoate (0.04 μCi) as described in the Materials and methods section. $^{14}\text{CO}_2$ production was measured in d.p.m., divided by the specific radioactivity of the medium (~ 700 d.p.m./nmol), and related to tissue weight. Samples (●) and (▲) were incubated in a final volume of 600 μl of standard assay buffer and sample (■) in standard assay buffer supplemented with 0.1% (w/v) Nonidet P40, 2 mM NAD^+ , 0.5 mM thiamin pyrophosphate and 0.8 mM CoA; the scale on the right ordinate applies to the latter set. Each value represents the mean $\pm$ S.E.M. for four to six observations.

chain oxo acid dehydrogenase activity (May *et al.*, 1980). Leupeptin and lipoamide dehydrogenase were not included in subsequent experiments.

Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin standards. Glucose was measured by the glucose oxidase- O_2 analyser (Beckman Instruments, Fullerton, CA, U.S.A.) method. Cytochrome *c* oxidase was assayed as previously described (May *et al.*, 1980). Thyroxine and T_3 were measured by radioimmunoassay (Immophase solid-phase separation system for T_3 and for thyroxine; Corning Medical and Scientific, Medfield, MA, U.S.A.).

Data are presented as means $\pm$ S.E.M. throughout the paper. Statistical analyses were performed by Student's *t* test or by the Mann-Whitney test when appropriate. Apparent Michaelis constants were determined by a weighted least-squares regression of the Lineweaver-Burk plot (Steel & Torrie, 1960).

Results

Effect of age (Table 1)

Significant age-dependent increases in the activity of the branched-chain oxo acid dehydrogenase were found (whether data are expressed per g wet wt. of liver or per mg of protein) in liver homogenates. The activity increased most rapidly during the perinatal period (2 days before and 1 day after birth) and gradually during the suckling period. There appeared to be a second spurt of increasing activity at the time of weaning (21 days), when near-adult values were

Table 1. Hepatic branched-chain oxo acid dehydrogenase activity as a function of age

The enzyme activity was measured in liver homogenates incubated at 37°C for 5 min with 0.2 mM 4-methyl-2-oxo[1- ^{14}C]pentanoate, as described in the Materials and methods section. Means $\pm$ S.E.M. are shown, for the numbers of animals indicated in parentheses. Foetal and weanling rats were not grouped according to sex. Adult rats were 1.5–3 months old.

Group	Branched-chain oxo acid decarboxylating activity		Protein content (mg/g of liver)
	(nmol/min per g of liver)	(nmol/min per mg of protein)	
Foetus (days gestation)			
20 (10)	16 ± 1.8	0.43 ± 0.10	47 ± 6.2
21 (8)	40 ± 2.8	0.41 ± 0.03	99 ± 6.0
22 (42)	67 ± 3.1	1.09 ± 0.08	71 ± 4.2
Postnatal (days)			
1 (12)	104 ± 19.5	0.95 ± 0.13	102 ± 12.0
2 (4)	96 ± 8.9	1.16 ± 0.14	83 ± 2.5
6–8 (14)	131 ± 8.1	1.29 ± 0.10	102 ± 3.1
14–17 (24)	121 ± 7.0	0.96 ± 0.05	125 ± 2.9
21 (18)	191 ± 10.9	1.70 ± 0.13	116 ± 4.0
Adult			
Male (13)	318 ± 23.6	2.68 ± 0.33	120 ± 7.0
Female (10)	245 ± 15.4	1.72 ± 0.10	142 ± 3.0
Pregnant female at term (6)	156 ± 10.2	1.14 ± 0.06	138 ± 6.2

reached. There was a more than 10-fold increase in enzyme activity per g of liver between day 20 of pregnancy (2 days *pre partum*) and the time of weaning. The increase was approx. 5-fold when expressed per mg of liver protein, owing to the concomitant increase in hepatic protein concentration (Dawkins, 1959).

In adult rats hepatic enzyme activity was greater in males than in females ($P < 0.01$), and was lower in pregnant dams at term (22 days) than in control females ($P < 0.01$). However, activity per g of liver in all adult rats exceeded foetal concentrations by at least an order of magnitude.

The dependence of enzyme activity on substrate concentration was measured in liver homogenates from foetal rats of 22 days gestation, weanling rats and adult male and female rats. Curvilinear Lineweaver-Burk plots (suggesting substrate inhibition) were obtained with standard assay conditions in all age groups, which precluded unambiguous assignment of K_m or V_{max} . Substrate inhibition of branched-chain 2-oxo acid dehydrogenase complex by high concentrations of 4-methyl-2-oxopentanoate has been reported (Parker & Randle, 1978). Enzyme activity at various concentrations of substrate was then measured in standard buffer supplemented with detergent and cofactors as described in the Materials and methods section. Nine concentrations of 4-methyl-2-oxopentanoate, between 5 and 2000 μM , were used; four liver homogenates were tested in each group. Under these conditions, the rate of oxidation of 4-methyl-2-oxopentanoate appeared to follow standard Michaelis-Menten kinetics in homogenates from both foetal (22 days gestation) and adult rats. The apparent V_{max} was 374 nmol of $^{14}CO_2$ /min per g tissue for the former and 1215 nmol/min per g for the latter. The K_m values were identical (130 μM). The decarboxylating

activity of the branched-chain 2-oxo acid dehydrogenase was enhanced approx. 2-fold in the presence of detergent.

Effect of maternal diabetes (Table 2)

The activity of the branched-chain 2-oxo acid dehydrogenase was significantly increased (whether data are expressed per g wet wt. of liver or per mg of protein) in liver homogenates from diabetic mothers. Maternal diabetes did not affect the hepatic enzyme activity in the newborns. Pups of diabetic mothers were hyperglycaemic at birth, although to a lesser degree than their mothers. Diabetic mothers gained less weight than did control mothers, and their pups were significantly smaller than those of controls [5.69 ± 0.12 g ($n = 24$) versus 6.07 ± 0.11 g ($n = 30$); $P < 0.05$].

Effect of maternal corticosterone administration (Table 2)

Steroid pretreatment of the mother had no effect on the decarboxylating activity (expressed per g of tissue) in homogenates from the offspring. Hepatic protein content increased slightly in pups of treated mothers, which accounts for the diminished specific activity (per mg of protein) of the dehydrogenase.

Effect of maternal T_3 administration

Dams who had received high doses of exogenous T_3 during the last 5 days of pregnancy had significantly increased plasma concentrations of T_3 [383.1 and 71.1 ng/dl ($n = 2$) versus 20.1 ± 3.9 mg/dl ($n = 3$); $P < 0.05$, Mann-Whitney test] and significantly decreased concentrations of thyroxine compared with controls [0.34 and 0.25 μg /dl ($n = 2$) versus 1.23 ± 0.04 μg /dl ($n = 3$); $P < 0.05$, Mann-Whitney test], indicating suppression of the pituitary-thyroid axis. Newborns in the control group

Table 2. *Effects of corticosterone or maternal diabetes on branched-chain oxo acid dehydrogenase activity*
Homogenates were prepared from the livers of foetuses of 22 days gestation whose mothers had been pretreated as described in the Materials and methods section. Mean values $\pm$ S.E.M. are given for the numbers of animals indicated.
^a $P < 0.05$ versus same-age control; ^b $P < 0.01$ versus same-age control; ^c $P < 0.01$ versus corresponding mothers.

Group	Branched-chain oxo acid decarboxylating activity		Protein content (mg/g of tissue)	Blood sugar (mg/dl)
	(nmol/min per g of liver)	(nmol/min per mg of protein)		
Mothers				
Control (3)	150 ± 11	1.2 ± 0.1	125 ± 4	81 ± 14
Diabetic (3)	306 ± 43 ^a	2.3 ± 0.1 ^b	133 ± 12	436 ± 35 ^b
Corticosterone (1)	147	1.2	126	123
Offspring of:				
Control (24)	67 ± 4 ^c	0.83 ± 0.04 ^c	86 ± 5 ^c	100 ± 6
Diabetic (20)	73 ± 5 ^c	0.83 ± 0.05 ^c	83 ± 4 ^c	220 ± 37
Corticosterone (6)	61 ± 11	0.60 ± 0.10 ^a	100 ± 5 ^a	105 ± 7

Table 3. *Effect of 3,3',5-tri-iodothyronine on hepatic protein content and activities of branched-chain oxo acid dehydrogenase and cytochrome c oxidase*

Liver homogenates were prepared from foetuses of 22 days gestation whose mothers were pretreated as described in the Materials and methods section. Means $\pm$ S.E.M. are shown for the number of observations indicated in parentheses. Each observation represents two or three pooled foetal livers. ^a $P < 0.05$ versus corresponding mother; ^b $P < 0.02$ versus same-age control; ^c $P < 0.001$ versus corresponding mother.

Group	Branched-chain oxo acid dehydrogenase (nmol/min per g of tissue)	Cytochrome <i>c</i> oxidase (μ mol of O ₂ /min per g of tissue)	Protein content (mg/g of tissue)
Control mothers	180 $\pm$ 6 (2)	10.5 $\pm$ 1.3 (3)	155 $\pm$ 4 (3)
Treated mothers	142 $\pm$ 5 (3) ^b	16.7 $\pm$ 0.2 (3) ^b	152 $\pm$ 11 (3)
Offspring of control	69 $\pm$ 4 (10) ^c	2.6 $\pm$ 0.2 (7) ^c	90 $\pm$ 2 (11)
Offspring of treated	82 $\pm$ 6 (10) ^c	4.1 $\pm$ 0.5 (7) ^{b,c}	123 $\pm$ 4 (10)

had significantly higher concentrations of thyroxine in plasma than those in the treated group ($0.93 \pm 0.08 \mu\text{g/dl}$ versus $0.50 \pm 0.14 \mu\text{g/dl}$; $n = 3$ per group; $P < 0.05$, t test). T₃ measurements were technically unreliable in the pups, presumably owing to more avid binding of T₃ by thyroxine-binding globulin (Braverman *et al.*, 1966).

Hepatic protein content and cytochrome *c* oxidase activity were significantly increased in pups from T₃-treated mothers (Table 3). Branched-chain oxo acid dehydrogenase activity was the same in both groups of pups. Pups of T₃-treated mothers were somewhat smaller than controls [$5.21 \pm 0.07 \text{ g}$ ($n = 21$) versus $5.63 \pm 0.06 \text{ g}$ ($n = 30$); $P < 0.001$]. There was, however, no correlation between body weight and the activities of either enzyme. Neonatal livers contained significantly less protein and lower activities of either enzyme than did those of the mothers (Table 3). The ratio of dehydrogenase activity/cytochrome *c* oxidase activity was greater in neonatal than in adult livers (Table 3).

Discussion

To our knowledge, there are no previous reports about the oxidation of leucine during the neonatal period, a time of rapid protein synthesis. The low branched-chain oxo acid dehydrogenase activity observed in foetal rat liver homogenates could be due to a number of factors, which include lack of enzyme, presence of an inactive form of the enzyme, lack of appropriate cofactors, presence of a mitochondrial transport barrier to substrate, or enzyme inhibition caused by intramitochondrial accumulation of products. The three last conditions would not explain the observation that liver homogenates prepared from term foetuses, treated with detergent and supplemented with cofactors, still exhibited much lower enzyme activities than did those from adults prepared under identical conditions. Since the apparent K_m was identical in preparations from

adult and foetal liver, it seems likely that the difference in V_{max} reflects lower enzyme content in the foetal group.

Foetal rat liver contains large amounts of erythropoietic tissue, which accounts for about 38% of the cells in the liver on day 20 of gestation and about 28% at birth (Finch & Thiel, 1964). The contribution of the haematopoietic system is smaller when based on liver weight rather than cell number (Greengard, 1971). However, even assuming that foetal haematopoietic tissue contributed to branched-chain oxo acid dehydrogenase activity, changes in hepatocellular composition could account for only a relatively minor portion of the observed increase in hepatic enzyme activity during development.

The observed apparent K_m was higher than previously reported for liver mitochondria (May *et al.*, 1980) or for the partially purified hepatic enzyme (Parker & Randle, 1978). This may be due to the presence in homogenates of a cytosolic decarboxylase, which has relatively low activity but a high K_m under the assay conditions used (May *et al.*, 1980; Sabourin & Bieber, 1981).

Increased hepatic activity of the dehydrogenase in adult diabetic rats (Shinnick & Harper, 1977; Paul & Adibi, 1978) is thought to represent mainly an increase in mitochondrial mass per g of tissue (May *et al.*, 1980). Foetal livers are known to contain less mitochondria than do those from adults (Dawkins, 1959; Hommes *et al.*, 1971). However, foetal rat livers did contain more branched-chain oxo acid dehydrogenase per cytochrome *c* oxidase than did adult livers (Table 3). The ratios reported here for adult livers are similar to previous observations (May *et al.*, 1980).

Diabetes also caused increased hepatic activity of the dehydrogenase in pregnant rats. However, although the altered metabolic milieu of the mother caused hyperglycaemia in the offspring, it did not affect foetal enzyme activity. Streptozotocin ad-

ministered to pregnant rats is not thought to deplete foetal insulin stores (Golob *et al.*, 1970).

Treatment with thyroxine decreases the lag time in perinatal expression of several hepatic enzymes, e.g. arginase (Greengard, 1971), aspartate aminotransferase (Herzfeld & Greengard, 1971), NADPH-cytochrome *c* dehydrogenase (Greengard & Dewey, 1968) and glucose 6-phosphatase (Greengard, 1969). Similarly, glucocorticoids are responsible for initiating the formation of other enzymes, e.g. UDP-glucose-glycogen glucosyltransferase, of the 'late foetal cluster' (for review see Greengard, 1971). We administered T_3 and corticosteroids in an attempt to hasten the induction of the branched-chain 2-oxo acid dehydrogenase. Although T_3 crosses the placenta poorly in primates (Pickering, 1968), significant transfer has been observed in rats (Van Wynsberghe & Noble, 1980). Evidence that this transfer occurred in our experiments includes the decrease in circulating foetal thyroxine concentration (reflecting pituitary suppression) and the parallel increase in hepatic cytochrome *c* oxidase activity in T_3 -treated mothers and their offspring. T_3 therapy has been reported to increase cytochrome *c* oxidase activity in soleus muscle of adult rats (Winder, 1979). However, treatment of the mother with pharmacological doses of either corticosterone or T_3 failed to increase the hepatic activity of the dehydrogenase in the newborn.

In adult rats the liver degrades considerable amounts of branched-chain 2-oxo acids, which are released by skeletal muscle into the circulation (Livesey & Lund, 1980). Thus low enzyme activity during foetal life would tend to preserve the carbon skeleton of branched-chain amino acids for protein synthesis. Hormonal manipulations which promote maternal protein catabolism failed to induce the enzyme prematurely in the foetus, further emphasizing the relative autonomy of foetal homeostasis.

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