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SPECIFIC DEVELOPMENT OF ISOCITRATE DEHYDROGENASES IN RAT BRAIN

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SUMMARY

Developmental changes of two different isocitrate dehydrogenases, NAD^+-linked (EC 1.1.1.41) and $NADP^+$-linked (EC 1.1.1.42) enzymes, were determined in various rat tissues. In most adult tissues, either cytoplasmic or mitochondrial $NADP^+$-linked enzyme showed the highest activity and NAD^+-linked enzyme showed the lowest. Whereas, brain NAD^+-linked enzyme activity predominated over both cytoplasmic and mitochondrial $NADP^+$-linked enzyme activities, and furthermore it was the highest in all tissues examined.

In adult brain, the higher activity was observed in the order of NAD^+-linked, mitochondrial $NADP^+$-linked and cytoplasmic $NADP^+$-linked enzymes. However, brain NAD^+-linked enzyme in newborn rats showed very low activity and cytoplasmic $NADP^+$-linked enzyme activity was higher in the newborn stage than in the adult stage. Drastic changes of the brain isocitrate dehydrogenase activities occurred between 5 and 20 days after birth. During this period, cytoplasmic $NADP^+$-linked enzyme decreased, mitochondrial $NADP^+$-linked enzyme maintained, and NAD^+-linked enzyme increased the activity. NAD^+-linked enzyme activity in liver and kidney remained at a low level during development.

INTRODUCTION

Most animal tissues contain two different isocitrate dehydrogenases, NAD^+-linked and $NADP^+$-linked enzymes. The latter have been found in mitochondrial and cytoplasmic fractions, and they can be separated by electrophoresis [1, 2]. Whereas, the NAD^+-linked enzyme is located exclusively in the mitochondria and is a typical allosteric enzyme, the activity varying with the concentration of nucleotides, and is activated by ADP and inhibited by ATP [3, 4, 5].

Developmental change of the cytoplasmic $NADP^+$-linked enzyme of rat brain occurs in a notable pattern where the activity decreases drastically during 20 days after birth [6, 7, 8]. This phenomenon is not consistent with the results of extensive studies that the most enzymes concerned with energy metabolism are several-fold higher in

activity at the adult stage compared with that at birth. Brain NAD^+-linked enzyme in some animals shows a higher activity than in other tissues [9]. This is thought to be significant for brain energy metabolism since the enzyme fulfils a key function in the citric acid cycle of the tissue and the activity is regulated by the ATP/ADP ratio. Recently, we have found [10] that bilirubin inhibits NAD^+-linked enzyme activity in vitro, but not $NADP^+$-linked enzyme and suggested that the pathogenesis of kernicterus may be related to the tissue-specific distribution of two isocitrate dehydrogenases. This paper describes the developmental and differentiated patterns of these isocitrate dehydrogenases in various rat tissues.

MATERIALS AND METHODS

Animals

Wistar rats at various stages were used. Breeding was carried out in our laboratory. Food and water were available ad libitum. Fetal rats were obtained from pregnant females the diagnosis of which was judged using a vaginal plug.

Chemicals

NAD^+, $NADP^+$ and ADP were obtained from Boerhinger–Mannheim Co. (GmbH). *threo*-D_SL_S-Isocitrate was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Enzyme assay

NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase activities were assayed by estimating the production of NAD(P)H at 340 nm [11, 12]. The initial reaction rates were measured in a Hitachi model 356 spectrophotometer at room temperature. The reaction mixture for the NAD^+-linked enzyme contained 33 mM Tris–acetate buffer at pH 7.2, 1.33 mM $MnCl_2$, 0.67 mM ADP, 0.33 mM NAD^+, 3.33 mM DL-isocitrate, water and enzyme, in a final volume of 3.0 ml. The reaction mixture for the $NADP^+$-linked enzyme contained 33 mM Tris–acetate buffer at pH 7.2, 1.33 mM $MnCl_2$, 0.05 mM $NADP^+$, 1.67 mM DL-isocitrate, 0.33 mM EDTA, water and enzyme, in a final volume of 3.0 ml. The reactions were carried out in silica cuvettes of 1-cm light path. Under these conditions, one unit of enzyme activity is defined as the amount of enzyme that forms 1 μmole of NAD(P)H per min.

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Pennington [13] with some modifications, using the microsomal, mitochondrial and nuclear suspensions as the enzyme sources.

Preparations of samples

The entire procedure was employed at 0–4 °C. Each tissue was homogenized with 10 vol. of 0.3 M mannitol containing 0.1 mM EDTA with a glass–Teflon homogenizer. The homogenized fluid was first centrifuged at 600 $\times$ g for 8 min to obtain the nuclear fraction. The supernatant was further centrifuged at 10 000 $\times$ g for 10 min. The pellet was used as the mitochondrial fraction. The 10 000 $\times$ g supernatant was further centrifuged at 100 000 $\times$ g for 1 h to remove microsomes from the cytoplasmic fraction. The nuclear, mitochondrial and microsomal pellets were suspended in adequate volumes of 0.3 M mannitol–EDTA solution after washing once by centri-

fugation. To solubilize and stabilize the NAD^+- and $NADP^+$-linked isocitrate dehydrogenases, an equal volume of a solution containing 10 mM potassium phosphate, 10% glycerol, 5 mM $MnCl_2$, 0.1 saturated $(NH_4)_2SO_4$ and 0.1% Triton X-100 at pH 7.2 was added to the nuclear, mitochondrial and microsomal suspensions and the resulting solutions were used as enzyme sources (Table I). When the various fractions

TABLE I

THE DISTRIBUTIONS OF NAD^+- AND $NADP^+$-LINKED ISOCITRATE DEHYDROGENASES AND SUCCINATE DEHYDROGENASE IN THE SUBCELLULAR FRACTIONS OF RAT TISSUES

Tissue	Age of animal (days)	Enzyme	Percentage of recovered activity			
			Cytoplasm	Microsomes	Mitochondria	Nuclei
Brain	2	NAD^+-linked enzyme	0	0	93.8	6.2
		$NADP^+$-linked enzyme	54.8	0.4	37.3	7.5
		Succinate dehydrogenase*	4.3	9.3	72.9	13.6
	70	NAD^+-linked enzyme	0	0	84.7	15.3
		$NADP^+$-linked enzyme	20.3	1.1	37.5	41.1
		Succinate dehydrogenase*	1.6	4.7	78.6	15.0
Liver	2	NAD^+-linked enzyme	0	0	33.3	66.7
		$NADP^+$-linked enzyme	50.2	5.4	15.5	28.9
		Succinate dehydrogenase	1.7	2.8	40.2	55.4
	70	NAD^+-linked enzyme	0	0	52.6	47.4
		$NADP^+$-linked enzyme	63.5	2.3	17.5	16.7
		Succinate dehydrogenase	1.7	3.2	55.4	39.7
Kidney	2	NAD^+-linked enzyme	0	0	52.6	47.4
		$NADP^+$-linked enzyme	43.3	0.4	20.2	36.1
		Succinate dehydrogenase	2.3	2.1	38.2	57.5
	70	NAD^+-linked enzyme	0	0	46.4	53.7
		$NADP^+$-linked enzyme	32.7	1.8	28.5	36.9
		Succinate dehydrogenase	1.2	3.9	51.3	43.6

* The succinate dehydrogenase activities in 2- and 70-day-old rat brains were 0.93 and 2.51 μmoles per min per g of wet weight, respectively.

of young and adult rat brains were assayed for NAD^+- and $NADP^+$-linked isocitrate dehydrogenases, NAD^+-linked enzyme activity was not detectable in the cytoplasmic or microsomal fractions and appeared mostly in the mitochondrial fractions. $NADP^+$-linked enzyme activity was shown in the cytoplasmic and also the mitochondrial or nuclear fraction; however, there was a very low activity in the microsomal fractions. The distribution of succinate dehydrogenase activity closely resembled that of the NAD^+-linked enzyme, but apparently differed from the $NADP^+$-linked enzyme. These results would be explained by the heterogenicity of brain mitochondria both in buoyant density and in enzyme content [14]. Liver and kidney were also fractionated by similar methods and the activities of two isocitrate dehydrogenases and succinate dehydrogenase were assayed. The succinate dehydrogenase activity was also used to determine the recovery of mitochondria by the method used in this experiment. Only about one-half or one-third of the total succinate dehydrogenase activity was recovered in the mitochondrial fractions. These results indicate the great contamination of

mitochondria in the nuclear fractions by these fractionations. Since the nuclear fractions contain high mitochondrial enzyme activities, these fractions were not separated from the mitochondrial fractions. In the succeeding experiments, the fractions, precipitated by centrifugation at 10 000 × *g* for 10 min from the original homogenates, were used for the assay of the mitochondrial enzyme activity.

RESULTS

When the isocitrate dehydrogenase activities of eight adult tissues were expressed per g of wet weight (Fig. 1), the total activity in liver, kidney and heart was

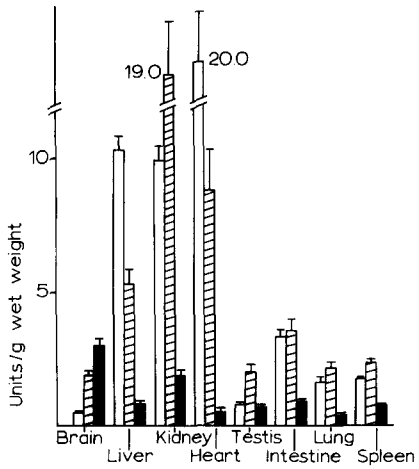

Fig. 1. A comparison of mitochondrial NAD^+-linked (filled bar), mitochondrial $NADP^+$-linked (hatched bar) and cytoplasmic $NADP^+$-linked (open bar) isocitrate dehydrogenase activities in eight tissues of adult rat. Each rectangle represents the mean value ± S.E., shown by the vertical line at the top of the rectangle. The enzyme preparations were derived from at least three animals.

higher than in brain. In most tissues, either cytoplasmic or mitochondrial $NADP^+$-linked enzyme showed the highest activity and NAD^+-linked enzyme showed the lowest. Brain NAD^+-linked enzyme activity predominated over both cytoplasmic and mitochondrial $NADP^+$-linked enzyme activities, and furthermore it was the highest in all tissues examined. In brain, the order of activity was NAD^+-linked enzyme, mitochondrial $NADP^+$- linked enzyme and cytoplasmic $NADP^+$-linked enzyme.

Mitochondrial NAD^+-linked and $NADP^+$-linked enzymes as well as cytoplasmic $NADP^+$-linked enzyme catalyze the dehydrogenation of isocitrate. However, the dehydrogenation linked to the citric acid cycle, electron transport and oxidative phosphorylation is actually carried out in mitochondria. In order to understand how the isocitrate dehydrogenation in mitochondria from each tissue depends on NAD^+-linked or $NADP^+$-linked enzyme, the ratio of NAD^+-linked and $NADP^+$-linked enzyme activities in the mitochondrial fractions was calculated (Table II). It showed that NAD^+-linked enzyme activity in brain occupied the greatest proportion with a ratio of 61.2%, whereas the total activity in other tissues was attributed mainly to $NADP^+$-linked enzyme with ratio of 72.7–94.2%.

TABLE II

THE RATIOS OF MITOCHONDRIAL NAD^+- AND $NADP^+$-LINKED ISOCITRATE DEHYDROGENASE ACTIVITIES IN EIGHT TISSUES OF ADULT RAT

Tissue	NAD^+-linked enzyme (%)	$NADP^+$-linked enzyme (%)
Brain	61.2	38.8
Liver	12.9	87.1
Kidney	9.1	90.9
Heart	5.8	94.2
Testis	27.3	72.7
Intestine	20.5	79.5
Lung	15.4	84.6
Spleen	24.4	75.6

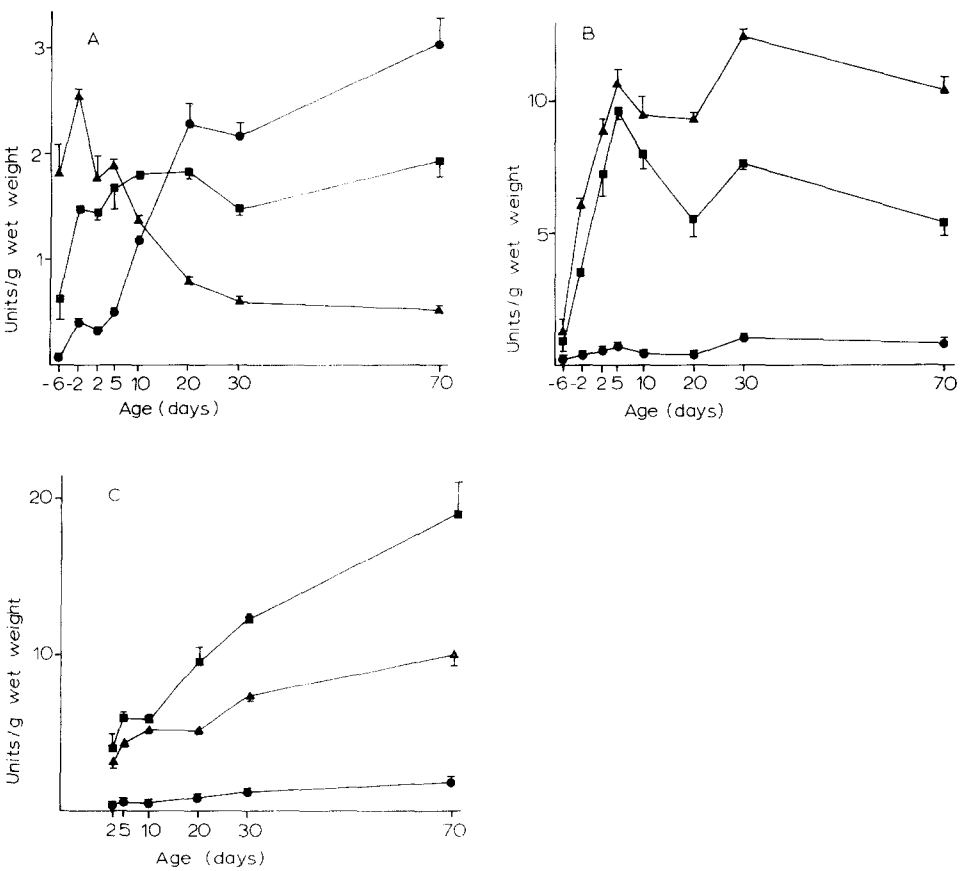

Fig. 2. Developmental changes of mitochondrial NAD^+-linked (●—●), mitochondrial $NADP^+$-linked (■—■) and cytoplasmic $NADP^+$-linked (▲—▲) isocitrate dehydrogenase activities in brain (A), liver (B) and kidney (C) of rat. Each age-point represents the mean value obtained for at least three animals. Standard errors are shown by vertical lines.

As shown in Figs 2A, B and C, the developmental patterns of isocitrate dehydrogenases were examined in brain, liver and kidney. The NAD^+-linked enzyme of brain showed very low activity in the newborn stage, in spite of the high activity in the adult stage, while cytoplasmic $NADP^+$-linked enzyme activity was higher in the newborn stage than in the adult stage. Drastic changes of the brain isocitrate dehydrogenase activities occurred between 5 and 20 days after birth. During this period, cytoplasmic $NADP^+$-linked enzyme activity decreased, mitochondrial $NADP^+$-linked enzyme maintained the enzyme level, and NAD^+-linked enzyme increased. The total isocitrate dehydrogenase activity of brain did not change predominantly with increasing age. In liver, cytoplasmic and mitochondrial $NADP^+$-linked enzymes showed a high activity in the newborn stage as well as in the adult stage, and the increase of both $NADP^+$-linked enzymes was observed in the last period of the neonatal stage. In kidney, both of the $NADP^+$-linked enzyme activities increased gradually from low levels at birth to higher levels in the adult stage. However, NAD^+-linked enzyme activity remained at a low level in liver and also in kidney during the development.

DISCUSSION

Isocitrate dehydrogenase is one of the enzymes of the citric acid cycle, but $NADP^+$-linked enzyme is not only located in mitochondria but also in cytoplasm. NAD^+-linked enzyme is included only in mitochondria. Loverde and Lehrer [15] reported in brain homogenates that NAD^+-linked enzyme activity was much higher in adult mice than in newborn mice and that $NADP^+$-linked enzyme activity was higher in newborn mice than in adult mice. Our study confirmed that the developmental patterns of the isocitrate dehydrogenases of cytoplasmic and mitochondrial fractions depicted particularly specific lines in brain, compared with those in liver and kidney. Between 5 and 20 days after birth, brain NAD^+-linked increased, mitochondrial $NADP^+$-linked enzyme maintained, and cytoplasmic $NADP^+$-linked enzyme decreased in activity. In newborn rats, brain cytoplasmic $NADP^+$-linked enzyme showed a high activity, as well as the other tissues of adult rat. This phenomenon means that the biochemical differentiation of brain has not occurred as yet at birth and NAD^+-linked enzyme and cytoplasmic $NADP^+$-linked enzyme are the competent marker enzymes dealing with the postnatal developmental process of the brain.

In adult brain, the NAD^+-linked enzyme activity was much higher than those of both the cytoplasmic and mitochondrial $NADP^+$-linked enzymes, whereas most tissues showed only little NAD^+-linked enzyme activity. The dehydrogenation by mitochondrial NAD^+-linked and $NADP^+$-linked enzymes linked to electron transport could be identical when $NADP^+$-linked enzyme is coupled with pyridine nucleotide transhydrogenase (EC 1.6.1.1). However, in brain, pyridine nucleotide transhydrogenase is not present in measurable amounts [9], suggesting that only NAD^+-linked enzyme would be used as ar dehydogenation enzyme linked to energy-yielding pathways. It is of interest that the greatest proportion of NAD^+-linked enzyme activity was seen in brain, because NAD^+-linked enzyme is an allosteric enzyme activated by ADP and inhibited by ATP, and, furthermore, is expected to function as a regulatory enzyme in the citric acid cycle of brain. The regulation of mitochondrial energy production and physiological function in brain are significantly interrelated. For example, the release of transmitter depends on the ATP produced by oxidative phosphorylation [16]. Sti-

mulation with electrical impulses or the increase of K^+ concentration caused increased respiration in mammalian cerebral cortex slices [17]. ADP is well-known to be linked to mitochondrial respiration; however, the specific dependence of brain mitochondria on the ATP/ADP ratio was not recognized. The increase of NAD^+-linked enzyme activity during development corresponds with the other development in brain, such as synapse formation [18], myelin synthesis [19], increase of $(Na^+–K^+)$-ATPase activity [20], and electrical spike appearance [21]. These morphological and physiological differentiations of brain may be interrelated with the basic metabolism capable of regulating the energy conditions, switch on or off.

Hyperbilirubinemia causes kernicterus; however, the brain-specific toxicity of bilirubin is not known. Recently, we found [10] that bilirubin was a potent inhibitor of NAD^+-linked isocitrate dehydrogenase, but not the $NADP^+$-linked enzyme. These results suggested that the brain energy metabolism would be selectively attacked by bilirubin, because other tissues have abundunt mitochondrial $NADP^+$-linked enzyme. Johnson et al. [22] reported that the occurrence of kernicterus and the death in inherited jaundiced rats (Gunn rats) were mostly seen in the third week after birth. This feature is also explained by the striking increase of NAD^+-linked enzyme activity in brain about 15 days after birth. Thus, the developmental studies of isocitrate dehydrogenases would propose an interesting problem with respect to brain metabolism, differentiation and malformation.

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