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AGE-SPECIFIC DEVELOPMENT OF MALATE-ASPARTATE SHUTTLE IN THE LIVER AND KIDNEY OF MICE

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Summary: The activities of malate-aspartate shuttle enzymes were measured in the liver and kidney of 15-, 30-, and 60-day old mice. The results indicate that the activities (U/mg protein) of both isoenzymes (cytosolic and mitochondrial) of both malate dehydrogenase and aspartate aminotransferase are significantly higher in the liver of 15-day old mice than in the liver of 30- and 60-day old animals. However, the shuttle enzymes showed a peak value in the kidney of 30-day old mice. *In vitro* reconstitution of malate-aspartate shuttle showed a similar pattern of activity in the tissues studied. These findings suggest that the activity of malate-aspartate shuttle is expressed differentially in these tissues of mice at different postnatal ages.

Introduction

The malate-aspartate shuttle appears to be the primary mechanism for the transfer of reducing equivalents from the cytosolic NADH to the mitochondria in many animal tissues (1-4). It has been shown that the inner mitochondrial membrane is quite impermeable to NADH. The NADH formed by glycolysis in the cytoplasm by the oxidation of glyceraldehyde 3-phosphate must be regenerated to NAD^+ for glycolysis to operate. The shuttle involves an influx of malate and glutamate and efflux of aspartate and α-ketoglutarate from the mitochondria (5-7). The main enzymes of the shuttle are malate dehydrogenase (MDH; EC 1.1.1.37) and aspartate aminotransferase (AsAT ; EC 2.6.1.1). Both these enzymes have two homologous and genetically independent isoenzymes: one in the cytosolic and the other in the mitochondrial fraction (8-11).

In developing animals metabolic adjustments take place in different tissues as an adaptation to the changing demands made upon them. Many studies have been done on individual enzymes as a function of age (12-16).

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But the reports on the activity of all the enzymes of a particular metabolic pathway are scanty (17).

In the present paper, we report a differential activity expression of malate-aspartate shuttle enzymes and the shuttle activity in the liver and kidney of mice at different postnatal ages.

Materials and Methods

Materials: Male Swiss albino mice (Balb/c strain) of three different age groups (15-, 30-, and 60-day old) were used. They were maintained under normal laboratory conditions at 24±2°C and fed soaked gram (*Cicer arietinum*), vitaminized milk powder, and tap water *ad libitum*. All the chemicals used were of analytical grade, and the biochemicals were obtained from Sigma Chemical Co., USA.

Preparation and assay of shuttle enzymes: Animals were killed by cervical dislocation at a fixed time of the day (11:00 h) and their livers and kidneys were taken out, washed in chilled normal saline, and blotted dry. Ten percent (w/v) homogenates of the liver and kidney were prepared in ice-cold 0.25 M sucrose. Each homogenate was centrifuged at 800 x g for 10 min at 0°C to sediment nuclei. The resulting supernatant was further centrifuged at 14,000 x g for 30 min at 0°C to sediment mitochondria. The supernatant thus obtained was used for the assay of cytosolic MDH and AsAT. The mitochondrial pellet was washed twice, suspended in a solubilizing medium (0.25 M sucrose/ 10 mM potassium phosphate buffer, pH 7.5/ 0.5% Triton X-100) for 3 h, and used for the assay of mitochondrial MDH and AsAT.

Both isoenzymes of both MDH and AsAT were assayed spectrophotometrically according to the method of Kitto (18) and Karmen, and Herzfeld and Greengard (19,20), respectively. Protein contents of the cytosolic and mitochondrial fractions were estimated (21), and the activity of both isoenzymes of both MDH and AsAT was expressed as Units (µmol NADH oxidized per min) per mg protein at 25°C.

Reconstitution of malate-aspartate shuttle: For the reconstitution of the malate-aspartate shuttle, the tissues were homogenized in 4 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4/ 0.25 M mannitol/ 1 mM EDTA/ 2 mM $MgCl_2$/ 30 mM 2-mercaptoethanol) and centrifuged at 800 x g for 10 min at 0°C to sediment nuclei. The supernatant was further centrifuged at 14,000 x g for 30 min to sediment mitochondria. The mitochondrial pellet was washed twice suspended in homogenization buffer, and used for the reconstitution assay. The post-mitochondrial supernatant was dialyzed for 18 h at 4°C against 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 2 mM $MgCl_2$, and 30 mM 2-mercaptoethanol. The dialyzed cytosol was centrifuged at 14,000 x g for 30 min to remove traces of particulate materials and the resultant clear supernatant was used for the reconstitution studies.

Reconstitution assay was done by the method of Cederbaum (1) and Dawson (22) with some modification of our own in the amount of protein taken and in the final assay volume. The reaction mixture (final volume, 2.5 ml) contained the following: buffer incubation medium (300 mM mannitol/ 10 mM potassium phosphate buffer, pH 7.4/ 10 mM Tris-HCl, pH 7.4/ 10 mM KCl/ 5 mM $MgCl_2$/ 2 mM ADP/ 2 mM aspartate), 2 mg cytosolic and 1 mg mitochondrial protein. After

setting of the baseline to zero, 0.05 ml of 7 mM NADH was added to the sample cuvette causing the absorbance of 0.70. The slow steady fall in absorbance was monitored for 2 min, and then 0.05 ml of a solution of 0.2 M each of L-malate and glutamate was added to both cuvettes. The decrease in absorbance was followed up to 10 min.

Results and Discussion

It is generally recognized that intact mitochondria are impermeable to externally added NADH (23). However, a considerable fraction of total NADH synthesized by the cell is made in the cytoplasm during glycolysis and the transport of reducing equivalent across the mitochondrial membrane is required for respiration and for a variety of metabolic processes. Various mechanisms for the transfer of reducing equivalents from the cytoplasm to the mitochondria have been proposed, of which glycerol phosphate and malate-aspartate shuttles are the most important (24-26). The most active NADH shuttle in the liver, kidney, and heart is the malate-aspartate one. The cytosolic and mitochondrial forms of malate dehydrogenase are key enzymes in the malate-aspartate shuttle. The cytosolic form catalyzes the oxidation of cytosolic NADH to NAD^+ with the concurrent reduction of oxaloacetate to malate. Malate thus formed is transported into the mitochondria where it is oxidized back to oxaloacetate by mitochondrial malate dehydrogenase with simultaneous reduction of NAD^+ to NADH. For the malate-aspartate shuttle to operate at a steady state, the rates of the mitochondrial and cytosolic dehydrogenases must be equal. In an attempt to assess all the enzymes of a particular metabolic pathway, we chose to study the activity expression of malate-aspartate shuttle and its enzymes in the liver and kidney of mice of different postnatal ages.

Figure 1a & b show that activities of both isoenzymes (cytosolic and mitochondrial) of both MDH and AsAT in the mouse liver were significantly higher at day 15, declined at day 30, and remained unchanged thereafter until the end of the experiment, at day 60. In contrast, the activities of these isoenzymes show a peak at day 30 in the kidney (Fig. 2a & b). It indicates an earlier developmental expression of shuttle enzymes in the liver than in the kidney of mice, which may in turn show an early involvement of malate-aspartate shuttle in the transfer of reducing equivalents to compensate the metabolic demand in the liver of growing mice. Interestingly, MDH isoenzymes showed a pattern of activity expression like that of AsAT isoenzymes in both tissues. Earlier reports showed that the rates

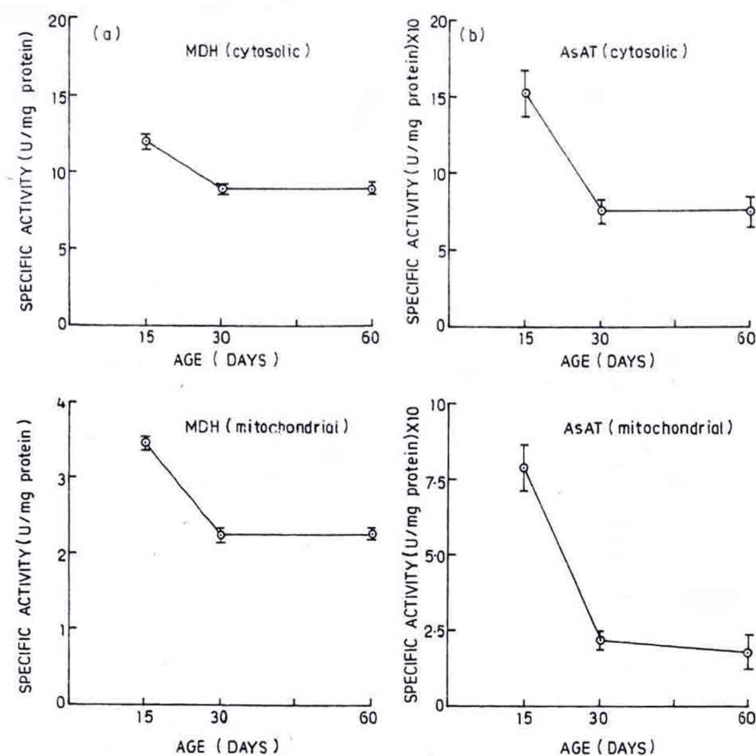


Fig. 1.(a) Activity of MDH isoenzymes (cytosolic and mitochondrial) in the liver of normal male mice of different postnatal ages. (b) Activity of AsAT isoenzymes (cytosolic and mitochondrial) in the liver of normal male mice of different postnatal ages. Fractionation and assay conditions are described in Materials and Methods. Values are means for 4-5 mice of each age group. Bars, S.D.

of the mitochondrial and cytosolic enzymes must be equal for the steady operation of this shuttle (27). Our findings are in agreement with the previous report that the AsAT develop differentially in different rat tissues (20). Unlike other aminotransferases, whose levels are insignificant in the fetal liver, the activity of AsAT expresses very early in the fetus about 4-5 days before birth and reaches a peak level by the second week of postnatal life in the rat liver (20).

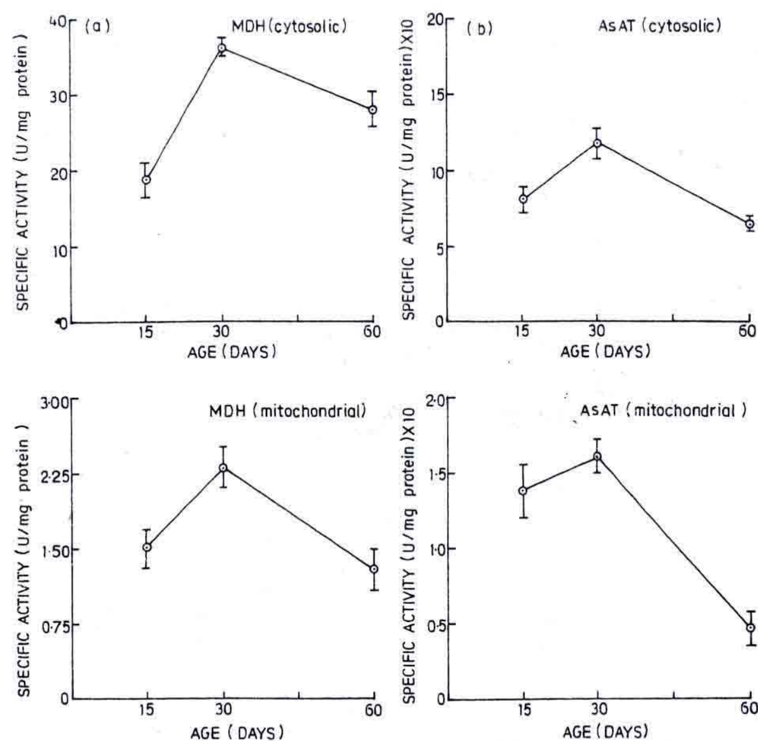


Fig. 2.(a) Activity of MDH isoenzymes in the kidney of normal male mice of different postnatal ages. (b) Activity of AsAT isoenzymes in the kidney of normal male mice at different postnatal ages. Experimental procedures are same as those of Fig, 1 a & b. Values are means from 4-5 mice of each age group. Bars, S.D.

In order to confirm the differential expression of malate-aspartate shuttle enzymes in the liver and kidney of mice, we studied the shuttle activity in a reconstituted system. Reconstitution of the malate-aspartate shuttle showed a higher activity (oxidation of NADH as measured by decrease in absorbance at 340 nm) in the liver of 15-day-old mice compared with that in 30-day-old animals (Fig. 3). Whereas, the shuttle activity was significantly higher in the kidney of 30-day-old mice than that in the kidney of

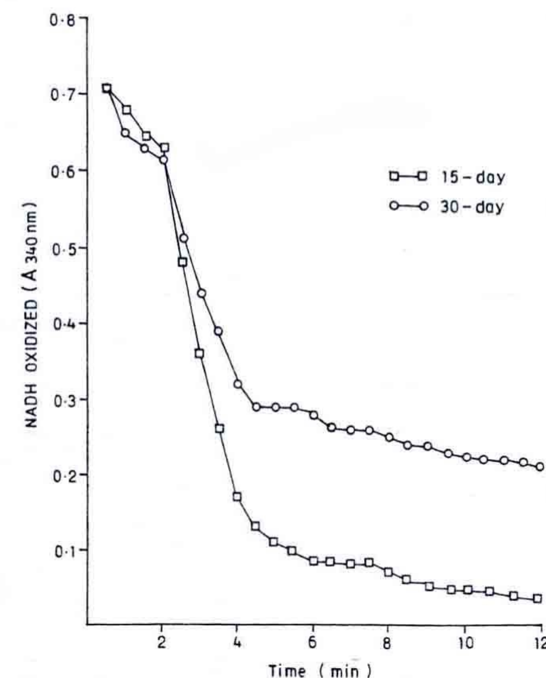
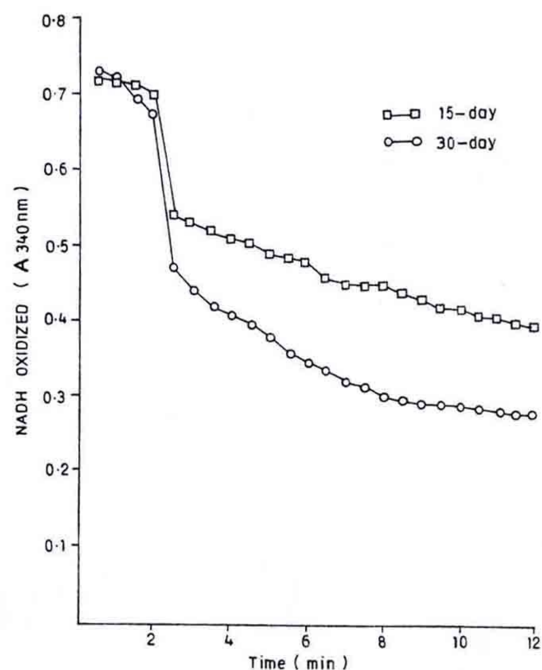


Fig. 3. Oxidation of NADH by reconstituted malate-aspartate shuttle in the liver of normal male mice. Equal amounts of dialyzed clear cytosol (2 mg protein) and mitochondria (1 mg protein) from both ages were used for the reconstitution assay. Preparation and incubation conditions are described in Materials and Methods. The traces depict the change in absorbance at 340 nm with the passage of time.

15-day-old ones (Fig. 4). Similar to the expression of enzymatic activities the shuttle activity showed an identical pattern in the liver and kidney of developing mice.

Our findings clearly indicate a differential expression of malate-aspartate shuttle in the liver and kidney of mice at different postnatal ages. This may reflect the differential metabolic transfer of reducing equivalents to compensate the different tissue requirements at various developmental ages.



ig. 4. Oxidation of NADH by reconstituted malate-aspartate shuttle in the idney of normal male mice. Experimental procedures were same as those of ig. 3. The traces depict the change in absorbance at 340 nm with the passage f time.

Acknowledgements

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