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Development of NADPH-Producing Pathways in Rat Heart

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The behaviours of the principal NADPH-producing enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, cytoplasmic and mitochondrial 'malic' enzyme and $NADP^+$-dependent isocitrate dehydrogenase) were studied during the development of rat heart and compared with those in brain and liver. 1. The enzymes belonging to the pentose phosphate pathway exhibit lower activities in heart than in other tissues throughout development. 2. The pattern of induction of heart cytoplasmic and mitochondrial 'malic' enzymes does not parallel that found in liver. Heart mitochondrial enzyme is slowly induced from birth onwards. 3. $NADP^+$-dependent isocitrate dehydrogenase has similar activities in all tissues in 18-day foetuses. 4. Heart mitochondrial $NADP^+$-dependent isocitrate dehydrogenase is greatly induced in the adult, where it attains a 10-fold higher activity than in liver. 5. The physiological functions of mitochondrial 'malic' enzyme and $NADP^+$-dependent isocitrate dehydrogenase are discussed.

Cellular NADPH is mainly produced by the oxidative pentose phosphate pathway. In eukaryotes there are two other enzymes capable of producing NADPH, 'malic' enzyme (EC 1.1.1.40) and $NADP^+$-dependent isocitrate dehydrogenase (EC 1.1.1.42). 'Malic' enzyme is considered to be an NADPH-producing enzyme in liver. It has also been postulated that it functions as part of a system transporting reducing equivalents in adrenal-cortex mitochondria (Simpson & Estabrook, 1969). However, the function of the mitochondrial enzyme in heart, which has an especially high activity, remains unknown.

Isocitrate dehydrogenase has a well-established role in prokaryotes, where it is present in only one form, normally the $NADP^+$-specific form. In eukaryotes, the presence of the two isocitrate-oxidizing enzymes, together with the dual compartmentalization of $NADP^+$-dependent isocitrate dehydrogenase in mitochondria and cytoplasm, has raised the yet-unresolved question of the actual role of this enzyme. As with 'malic' enzyme, $NADP^+$-dependent isocitrate dehydrogenase has very high activity in adult rat heart. Although it might function coupled to the energy-independent transhydrogenase to bring about isocitrate oxidation, the relative activities of the two heart enzymes only allow the coupling of a fraction of total $NADP^+$-dependent isocitrate dehydrogenase to transhydrogenase (Stein *et al.*, 1967). We have tried to gain new insight into the function of these two enzymes ('malic' enzyme and $NADP^+$-dependent isocitrate dehydrogenase) by studying their developmental patterns in three organs of different metabolic characteristics (liver, heart and brain). As a control for behaviour of classical NADPH-producing enzymes, the variations in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have also been recorded.

Materials and Methods

Chemicals

$NADP^+$, DL-isocitrate, L-malate and 6-phosphogluconate were all purchased from Sigma Chemical Co. Glucose 6-phosphate was from Boehringer Corp. Other reagents were of the highest purity available from commercial sources.

Preparation of tissue extracts

Male and female Wistar rats were used. Fresh tissues were homogenized in a 7-fold volume of ice-cold medium containing 0.33 M-sucrose, 0.05 mM-EDTA and 15 mM-Tris/HCl, pH 7.4, with ten strokes of an all-glass Potter–Elvehjem homogenizer.

For separate determinations of extra- and intra-mitochondrial enzyme activities, rat liver and heart mitochondrial fractions were prepared by the method of Carvalho (1974). Mitochondria from brain were prepared as described by Clark &

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Nicklas (1970). Supernatants II, obtained after the first 8200*g* centrifugation of liver and heart extracts, were further centrifuged at 8200*g* and the resultant supernatants were used as cytoplasmic fractions. Supernatant II resulting from the first 12500*g* centrifugation of brain extracts was further spun at 11500*g* and the supernatant obtained was used as brain cytoplasmic fraction. Submitochondrial particles were obtained by ultrasonic low-frequency (3μm) treatment of mitochondrial preparations in four periods of 30s each. Heating was prevented by interrupting each period for 15s. An MSE 150W ultrasonic sonicator with refrigerating compartment was used. Mitochondrial contamination of cytoplasmic fractions was determined through the assay of citrate synthase, used as a mitochondrial marker enzyme. In all the experiments recorded, this contamination did not exceed 10%.

Measurements of enzyme activities

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were determinated as described by Sapag-Hagar *et al.* (1973). 'Malic' enzyme (decarboxylating) was assayed essentially by the method of Ochoa *et al.* (1948). The reaction mixture consisted of 100mM-Tris/HCl, pH7.4, 1mM-$MnCl_2$, 0.25mM-$NADP^+$, 1.5mM-L-malate and extract in a total volume of 1.0ml. $NADP^+$-dependent isocitrate dehydrogenase activity was measured as described by Plaut & Aogaichi (1968). The rate of NADPH formation was recorded at 340nm and 25°C. Citrate synthase was determined as previously described (Núñez de Castro *et al.*, 1976). Enzyme activities are given as munits/mg of protein, where 1munit of activity is taken as that amount of enzyme that transforms 1nmol of substrate in 1min at 25°C. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electrophoresis

Vertical starch-gel (12%, w/v) electrophoresis was carried out at 4°C for 6–7h. The buffer systems used in the preparation of the gels were 25mM-sodium borate/NaOH, pH8.6, and 25mM-sodium borate/HCl, pH6.0. The bridge buffers were 0.1M-sodium borate/NaOH, pH8.6, and 0.1M-sodium citrate, pH6.0, respectively, both buffers containing 0.2mM-DL-isocitrate. The pH8.6 gels were run at 22V/cm for 7h, and the pH6.0 gels at 11V/cm for 6h. $NADP^+$-dependent isocitrate dehydrogenase isoenzymes were stained with 5mM-$MnCl_2$/4.5mM-DL-isocitrate/0.5mM-$NADP^+$/Nitro Blue Tetrazolium (1mg/ml)/phenazine methosulphate (0.3mg/ml)/0.1M-Tris/HCl, pH8.0. The gels were incubated at 37°C in the dark until the bands were clearly visible.

Results and Discussion

Oxidative pentose phosphate pathway

The patterns of variation of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities with growth are shown in Figs. 1 and 2. Our results for the liver and brain enzymes

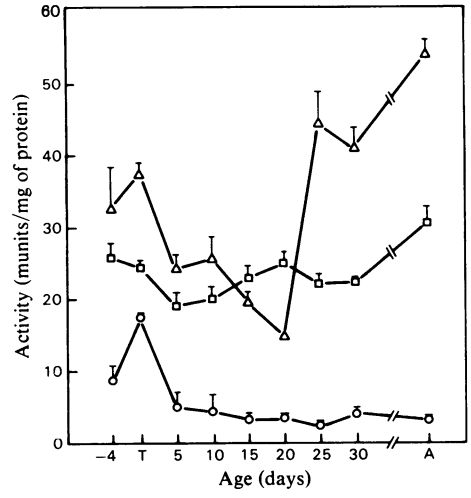

Fig. 1. *Development of cytoplasmic glucose 6-phosphate dehydrogenase in heart* (○), *liver* (△) *and brain* (□) Tissue extracts and cellular fractions were obtained and assayed for activity as described in the Materials and Methods section. The results are means ± S.E.M. for five experiments. Abbreviations: T, term; A, adult.

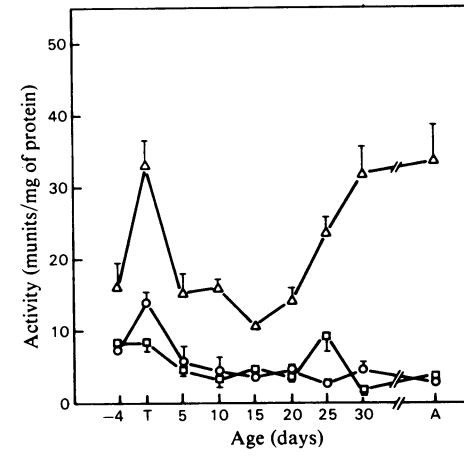

Fig. 2. *Development of cytoplasmic 6-phosphogluconate dehydrogenase in heart* (○), *liver* (△) *and brain* (□) For details see the legend to Fig. 1.

are in agreement with the NADPH-producing role of the pathway and with those obtained by Vernon & Walker (1968*a*,*b*). Both enzymes show a pronounced increase in activity around birth followed by a decrease 5 days afterwards, both in liver and heart. Such a pattern is not observed in brain.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are highest in liver, in agreement with the lipogenic character of this organ; their activities are lowest in heart. Two different explanations could account for this low activity: (*a*) the NADPH needs of the heart are relatively low and are covered by the oxidative pentose pathway; (*b*) NADPH production in heart is brought about by other enzymes besides that of the pentose phosphate pathway, namely 'malic' enzyme and/or $NADP^+$-linked isocitrate dehydrogenase.

'Malic' enzyme

Figs. 3 and 4 show the patterns of variation of cytoplasmic and mitochondrial 'malic' enzyme activities in heart, liver and brain. As Vernon & Walker (1968*a*) have previously reported, a strong induction of the liver cytoplasmic enzyme is produced around the 20th day, at the time of weaning. Keeping pace with it, the brain and heart enzymes are also induced, though to a minor extent.

Since the increase at weaning of the classical NADPH-producing pathways is related to their lipogenic role, liver 'malic' enzyme is generally accepted as being an enzyme that functions during lipid biosynthesis. The increase in heart mitochondrial 'malic' enzyme activity cannot be explained as being a response to lipogenic conditions because (*a*) the activity of this enzyme gradually increases throughout development and does not exhibit a marked increase at weaning, and (*b*) the pentose phosphate pathway, which meets NADPH demands in liver, does not increase in heart.

On the other hand, the unusually low pyruvate carboxylase activity (Ballard *et al.*, 1970) compared with that of liver, kidney and adipose tissue raises the question of the origin of the dicarboxylic acids needed for fatty acid oxidation. Moreover, pyruvate utilization by heart through the classical pyruvate-using pathways simultaneously with that of fatty acids does not seem favoured in the light of the following: (*a*) the above-mentioned virtual absence of pyruvate carboxylase; (*b*) the inhibition of pyruvate dehydrogenase by acyl-CoA of any number of carbon atoms (Olson *et al.*, 1978); (*c*) the kinetic characteristics of heart lactate dehydrogenase (low activity for pyruvate reduction, high K_m for pyruvate, inhibition by excess pyruvate) that normally prevent lactate production in heart (Kaplan *et al.*, 1968). Lowenstein (1972) has proposed a purine nucleotide cycle that would provide dicarboxylic acids from aspartate and glutamate for replenishment of the tricarboxylic acid cycle. Yet another way of producing tricarboxylic acid-cycle intermediates, especially under conditions in which fatty acids plus pyruvate are utilized, could involve pyruvate carboxylation through 'malic' enzyme. Accumulation of pyruvate as a result of the heart's inability to metabolize it at a rapid rate would shift the activity of 'malic' enzyme towards malic acid production ($\Delta G'^0 - 1.46\,kJ/mol$). Heart NADPH concentrations may be especially high as a result of the increased mitochondrial $NADP^+$-linked isocitrate dehydrogenase activity, which has a K_i for NADPH one order of magnitude higher than that of the liver enzyme (A. Andrés, J. Satrústegui & A. Machado, unpublished results). The once-

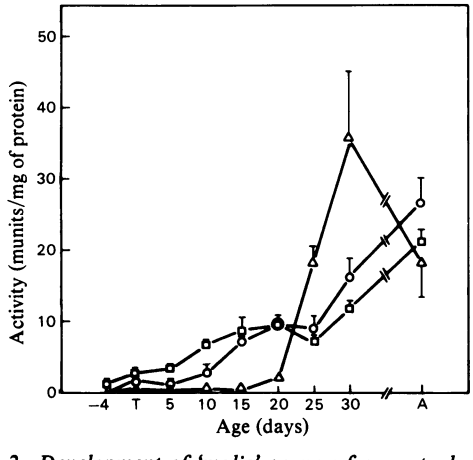

Fig. 3. *Development of 'malic' enzyme from cytoplasmic fractions of heart* (○), *liver* (△) *and brain* (□)
For details see the legend to Fig. 1.

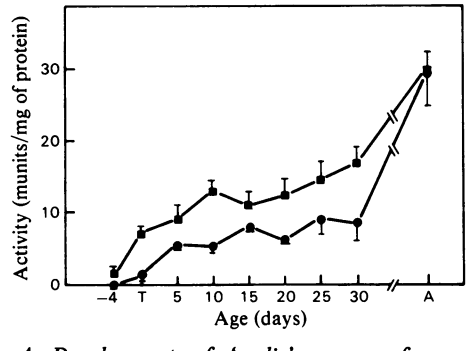

Fig. 4. *Development of 'malic' enzyme from mitochondrial fractions of heart* (●) *and brain* (■)
For details see the legend to Fig. 1.

postulated and rejected (Frenkel, 1975) anaplerotic role for 'malic' enzyme might, in heart at least, again become an open question.

$NADP^+$-linked isocitrate dehydrogenase

(*a*) *Isoenzyme pattern.* In order to study the development of $NADP^+$-dependent isocitrate dehydrogenase, the electrophoretic behaviour of the foetal and adult isoenzymes was compared. All three organs studied had two isoenzymes corresponding to the mitochondrial and cytoplasmic fractions respectively, with different relative mobilities, as described by Fox & Crockett (1977) and Henderson (1968) for adult mouse tissues. The rat mitochondrial isoenzymes of the three adult and foetal organs had the same mobility, and migrated towards the cathode at both pH 6.0 and 8.6. Adult and foetal cytoplasmic isoenzymes from heart, liver and brain also had the same mobility and migrated towards the anode at the same pH values. The cytoplasmic and mitochondrial isoenzymes also had different stabilities. The cytoplasmic isoenzyme was resistant to several freezing/thawing cycles and to maintenance at 14°C for 24h, whereas the mitochondrial isoenzyme was greatly inactivated by both conditions (33% loss after 24h at 14°C).

(*b*) *Developmental pattern.* $NADP^+$-dependent isocitrate dehydrogenase shows a clearly distinct pattern of variation in each of the tissues studied (Figs. 5 and 6). The remarkable similarity between the mitochondrial enzyme activities of all three organs in foetuses is in sharp contrast with the large differences that develop between them during growth. The heart enzyme increases 5–6 times in activity, whereas the liver and brain enzymes decrease to 60 and 18% of their initial values respectively. Such behaviour could reflect the physiological significance of $NADP^+$-linked isocitrate oxidation in the different tissues. If this is the case, isocitrate oxidation coupled to NADPH production is a pathway of overwhelming importance in heart mitochondria, and the low activity in brain mitochondria can be regarded as an evolutionary residue. The importance of the liver mitochondrial enzyme is also doubtful. Cytoplasmic $NADP^+$-dependent isocitrate dehydrogenase is subject to a small but rapid induction in liver, around the third day before birth, in contrast with the behaviour of the enzyme in other organs. This result differs from those found by Vernon & Walker (1968*a*), who did not observe any appreciable variation in enzyme activities during development. The failure to observe the small cytoplasmic induction could be the result of assaying the activity in whole extracts, where the balanced changes in activity of both compartments could mask the individual patterns. A possible role for cytoplasmic $NADP^+$-dependent isocitrate dehydrogenase could be NADPH production for lipogenesis. This is, however, unlikely, since the enzyme-developmental pattern does not parallel those of the pentose phosphate pathways and 'malic' enzyme after the diet change at weaning. Furthermore, cytoplasmic isocitrate dehydrogenase is probably inhibited under conditions of lipogenesis, since isocitrate oxidation competes with citrate utilization for acetyl-CoA formation.

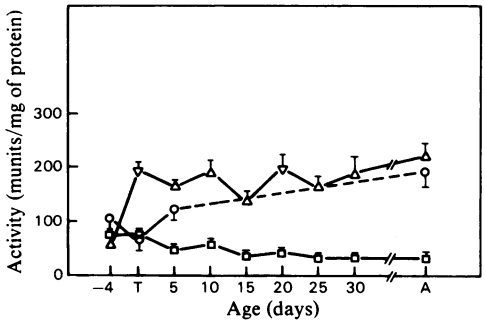

Fig. 5 *Development of $NADP^+$-linked isocitrate dehydrogenase from cytoplasmic fractions of heart* (○), *liver* (△) *and brain* (□)
For details see the legend to Fig. 1.

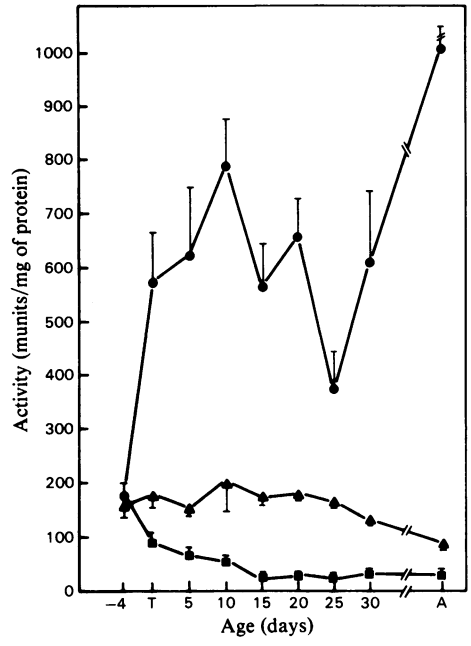

Fig. 6. *Development of $NADP^+$-linked isocitrate dehydrogenase from mitochondrial fractions of heart* (●), *liver* (▲) *and brain* (■)
For details see the legend to Fig. 1.

As for the role of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase, few explanations have been put forward. It has been suggested that it could function in isocitrate oxidation through NADPH/NAD^+ transhydrogenase (Stein *et al.*, 1967). However, in heart the actual transhydrogenase activity could be coupled to no more than a fraction of the total NADPH produced through $NADP^+$-dependent isocitrate dehydrogenase (Chico *et al.*, 1977).

We suggest that the function of $NADP^+$-dependent isocitrate dehydrogenase in heart mitochondria is probably the same throughout development and is that of foetal hearts, namely isocitrate oxidation under oxygen deficiency, when a high NADH/NAD^+ ratio brings about a virtual stop in NADH consumption through respiration and a block in all NADH-producing steps. The increase in $NADP^+$-dependent isocitrate dehydrogenase during heart development would keep pace with the need of an hypoxic-resistant pathway upon which the adult viability would depend under oxygen-limiting conditions.

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